JOURNAL OF VIROLOGY, Apr., 1989, p. 1704–1714
0022-538X/89/041704-11$02.00/0

Vol. 63, No. 4

Effects of Cell Differentiation on Replication of A/WS/33, WSN, and A/PR/8/34 Influenza Viruses in Mouse Brain Cell Cultures: Biological and Immunological Characterization of Products

GARY L. BRADSHAW, R. WALTER SCHLESINGER,* AND CHARLES D. SCHWARTZ

Department of Molecular Genetics and Microbiology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854-5635

Received 20 September 1988/Accepted 21 December 1988

The responses of mouse embryo brain (MEB) cell cultures and of Madin-Darby canine kidney cells and chicken embryo fibroblasts to infection with A/PR/8/34 (PR8), A/WS/33 (WS), or the neurovirulent WSN variant were compared in terms of (i) single-cycle yields of hemagglutinating and associated neuraminidase (NA) activities and plaque-forming particles, the latter with or without trypsin activation [PFU(TR++) or PFU(TR−−), respectively], and (ii) expression of nucleoprotein (NP), M1, and NS1 protein, determined for specific cell types by immunostaining, for whole culture lysates by Western blot analysis of NP and M1. Primary MEB cultures grown in serum-enriched medium were infected after 6 days (young), when none of the cells reacted specifically and exclusively with any of the nerve cell marker antibodies used, or after ≥21 days (aged), when astrocytes (the predominant cell type), neurons, and oligodendrocytes were morphologically and immunologically mature. Secondary astrocyte-enriched cultures were used when they contained 90 to 99% of their cells as astrocytes at an early stage of differentiation. By all criteria, young MEB cultures were only marginally less permissive for each of the three viruses than were chicken embryo fibroblasts or Madin-Darby canine kidney cells. Aged MEB cultures, by comparison, produced undiminished NP, hemagglutinin, and neuraminidase, but yields of PFU(TR++) and expression of M1 protein (relative to NP) were reduced for all three viruses, most for PR8 and least for WSN; relative reduction of NS1 protein was demonstrable only in PR8-infected aged cultures. Immunostaining revealed low levels of M1 and NS1 expression only in astrocytes, not in oligodendrocytes and neurons. In PR8-infected mature astrocytes, NP accumulated in the nucleus; it persisted in some cells for at least 8 weeks after infection. The presence of NP did not seem to interfere with cell division. Secondary MEB cultures containing 90 to 99% immature astrocytes were less restricted than were aged primary cultures. Thus, it appears that reduced permissivity of nerve cell cultures, as measured in this study, is most closely correlated with advancing differentiation and maturity of astroglial cells. Assembled virions, including those that score as PFU(TR++) in restricted cultures (e.g., PR8-infected aged MEB), may be mainly products of mature oligodendrocytes and neurons.

As far as we are aware, the only human influenza viruses from which variants expressing neurovirulence in mice have ever been derived directly (i.e., not by reassortment) are the early H1N1 strains A/WS/33 (WS) and A/Mel/35; the latter variant seems to have been maintained only for a limited number of passages in minced chicken embryo brain cultures (7). In contrast, the WS variants NWS (24) and WSN (7) have been maintained in many laboratories around the world and have retained their unique pathogenic properties. Since the late 1940s, they have served as donors of genes determining the neurovirulent phenotype for seminal studies of recombination (reviewed in reference 25). This approach has led to identification of three WSN RNA segments which must be present in WSN × A/Aichi/2/68 (H3N2) reassortments to permit expression of full in vivo neurovirulence (26) and multiple replication cycles in brains as well as in cultured murine neuroblastoma cells (16). These are WSN gene segments 6 (coding for neuraminidase [NA]), 7 (M1 and M2 proteins), and 8 (NS1 and NS2 proteins).

Is the ability to generate stably neurovirulent variants unique to the first human strain of influenza virus ever isolated (WS) (23) and, perhaps, A/Mel/35? It is unlikely that comparably determined efforts have been made with other H1N1 strains or other subtypes. Comparative gene sequence analyses are thus far limited to WSN and PR8 segments 1, 2, 4, 6 (14), and part of 7 (2), but the parental WS strain has not yet been sequenced. Lacking insights from sequence data, we are asking how expression of gene segments 6, 7, and 8 of non-neuroadapted H1N1 strains, such as egg-adapted parental WS or A/PR/8/34 (PR8), in mouse nerve cells differs from that of the analogous WSN genes.

In a companion article (20), we describe the first in vivo experiments done with the aim of reexamining the nature of what was characterized many years ago as incomplete replication of egg-adapted, non-neurovirulent H1N1 strains in mouse brains (18, 19, 27). We confirmed that replication of the WS and PR8 strains after intracerebral (i.c.) inoculation is restricted to a single cycle, presumably because of failure of cleavage of hemagglutinin (HA) into its subunits, HA1 and HA2. In the case of WSN and its neurovirulent recombinants, cleavage is thought to be indirectly facilitated by some function unique to WSN NA (21, 22). However, possession of gene segment 6 as the only contribution of WSN does not endow reassortants with neurovirulence (26). Therefore, we are concentrating on the expression of one or more of the four gene products of WS and PR8 RNA segments 7 and 8 in

* Corresponding author.

an effort to explain the relatively low yields of trypsin-activated progeny virions and lack of virulence in mice after i.c. infection with these two strains.

Our findings suggest that expression of M1 protein is indeed restricted in brains of mice infected with PR8 compared with WSN-infected brains. Unfortunately, in vivo experiments do not allow separation of cell-associated from released products of viral replication or use of radioactive labeling. Moreover, possible differences in expression of M2 as well as NS1 and NS2 proteins would be difficult to assess in the living host. Since these three proteins are encoded in gene segments 7 or 8, respectively, their strain-specific effects have to be considered, along with those of NA and M1 protein.

These considerations have led us to develop nerve cell cultures as model systems for more meaningful approaches to future in vivo studies. Here we describe the establishment of such cultures from mouse embryo brains (MEB) and identification of cell types represented after varying periods of in vitro differentiation. We report virus strain-specific differences between certain specialized cell types with regard to viral replication and gene expression, as measured by biological and immunological criteria. A brief preliminary summary of initial findings was published in a conference report (3).

MATERIALS AND METHODS

Viruses, preparation of stock virus, and plaque assays. The WS and PR8 strains and the neurotropic WSN variant of H1N1 type A influenza virus, their passage histories in our laboratory, preparation of virus stocks, cultures of Madin-Darby canine kidney (MDCK) cells and chicken embryo fibroblasts (CEF), and use of these cells in plaque assays have been previously described (20). All experiments involving WSN virus were done with a single stock prepared by one additional passage in baby mice, followed by intraallantoic transfer of brain homogenate at limiting dilution. Results of plaque assays (PFU per milliliter or per number of cells) are expressed as PFU(TR−−) (virus sample not pretreated with trypsin, no trypsin added to overlay medium), PFU(TR−+) (sample not pretreated with trypsin; *p*-tosyl-L-phenylalanine chloromethyl ketone hydrochloride-treated trypsin [TPCK-TR], 2 μg/ml, added to overlay medium), or PFU(TR++) (sample pretreated with TPCK-TR, 10 μg/ml, for 20 min at 37°C, followed by addition of TPCK-TR, 2 μg/ml, to overlay medium).

HA particles. The estimated number of hemagglutinating particles (HA particles) per unit volume or per number of cells is expressed as (reciprocal of HA titer) × (number of fowl erythrocytes/ml) (27).

NA assays. NA assays were done by incubating samples with nonlimiting concentrations of fetuin, predetermined by kinetic assays, for various periods at 37°C. Released *N*-acetylneuraminic acid was quantitated by the method of Aminoff (1).

Primary MEB cell cultures. Embryos of Swiss Webster mice (Perfection Breeders, Douglasville, Pa.) were collected on days 17 to 20 of gestation. Whole brains were removed and dispersed through nylon mesh of approximately 50-μm pore size as described by Honegger et al. (10). The dispersed cells were collected into serum-free Dulbecco modified Eagle medium (DME) (D-MEM powder with D-glucose, 4.5 g/liter; 1987 catalogue no. 430-2100; GIBCO Laboratories, Grand Island, N.Y.) and centrifuged at 600 × *g* for 5 min. The cells, resuspended in DME containing 10% horse serum, were plated at a density of 0.5×10^5 to 1×10^5 cells per cm^2 and incubated at 37°C in a 10% CO_2 atmosphere, with medium changes every 3 to 4 days. The characteristics of the cultures after various periods of growth are described in Results.

Secondary cell cultures. Secondary astrocyte-enriched (AE) MEB cell cultures were prepared according to the method of Fischer et al. (6), with minor modifications. Dispersed cells, obtained as above, were plated in serum-free DME supplemented with growth factors as described by Fischer et al. (6) except for omission of aprotinin. After 2 weeks, the cells were released from the flasks with 5 mM EDTA in DME and subcultured in serum-free DME on poly-L-lysine-coated plastic or glass substrata. Three days later, serum-free DME was replaced with DME containing 10% horse serum. All incubations were at 37°C in a 10% CO_2 incubator. The characteristics of these secondary cultures are described in Results.

Infection of cell cultures. Allantoic fluid stock virus, diluted in phosphate-buffered saline containing 0.2% bovine serum albumin, was inoculated onto cell sheets (approximately 600 μl/cm^2) to give the desired input multiplicity of infection (MOI). After adsorption for 1 h at 37°C, cultures were washed three times with phosphate-buffered saline, and then serum-free DME was added in a volume of 100 to 200 μl/cm^2, marking time zero of the experiment. The cultures were then further incubated at 37°C in a 10% CO_2 atmosphere.

Harvest and assay of infectious and HA particles from cell cultures. Media were centrifuged at 10,000 × *g* for 1 min and made 0.2% with bovine serum albumin. Cell sheets were scraped into equivalent volumes of Tris-buffered saline (TBS; 0.01 M Tris [pH 7.4], 0.15 M NaCl); media and cell suspensions were frozen at −80°C. For assays, cell suspensions were thawed and then frozen and thawed twice and centrifuged at 900 × *g* for 15 to 20 min. Plaque assays on MDCK cell monolayers and HA titrations as well as concentration of HA particles by hemadsorption and elution, sometimes followed by pelleting, were all done as described in the companion article (20).

Staining. For immunostaining, cell cultures on cover slips were washed twice with TBS and fixed at room temperature with 10% Formalin in TBS for 30 min, then for another 30 min with absolute methanol containing 0.3% H_2O_2. Three washes with TBS were followed by incubation at room temperature with blocking buffer (TBS containing 5% normal goat serum), which was replaced after 30 min by primary antibody diluted in blocking buffer containing 0.01% sodium azide. After overnight incubation at 4°C and three 10-min washes with TBS, the cultures were incubated with either goat anti-rabbit or peroxidase-conjugated anti-mouse immunoglobulin G, each diluted 1:40 in blocking buffer. Cultures reacted with polyclonal rabbit antiserum as the primary antibody were further incubated with peroxidase-antiperoxidase diluted 50-fold in blocking buffer and washed again. Immunoreactive cellular or viral proteins were localized by a 1- to 2-min incubation at room temperature with 0.05 M Tris hydrochloride (pH 7.4) containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2. Counterstain was hematoxylin.

For double immunostaining of cellular and viral proteins, cultures were reacted with appropriate monoclonal and/or polyclonal antibodies as described above. The order in which the antibodies were added and the method of the alternate disclosing reaction with glucose oxidase and anti-glucose oxidase followed the description by Clark et al. (4).

Other procedures. Sodium dodecyl sulfate-polyacrylamide

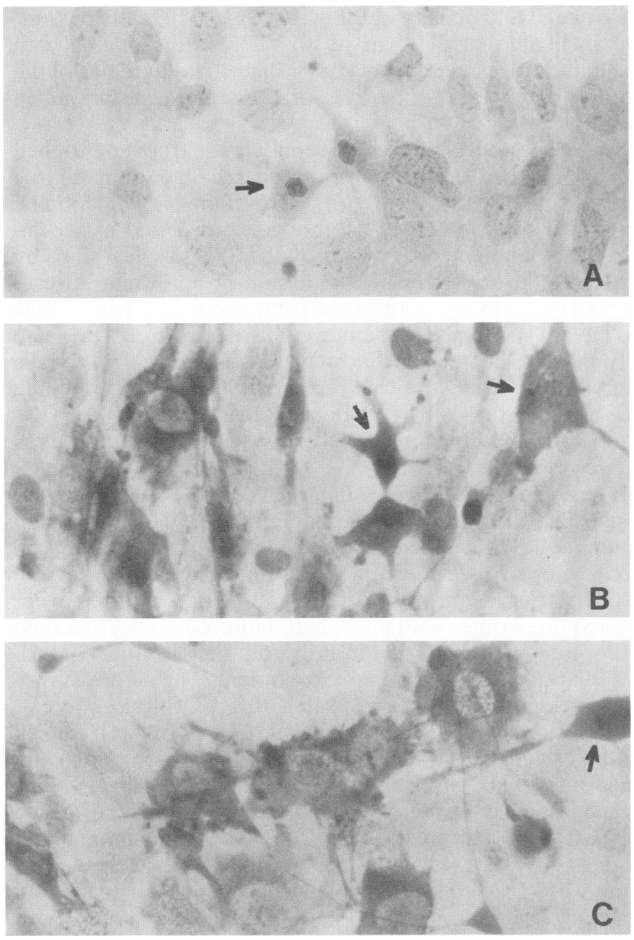

FIG. 1. Mock-infected (A) and PR8-infected (MOI = 0.1 PFU per cell) (B and C) 6-day-old MEB cell cultures 6 h p.i. (A and B) Reactions with polyclonal anti-NP X-73, WN11A and (C) with anti-M1 3735, final HPLC, immune sera, both supplied by K. van Wyke-Coelingh. Reactions were localized with peroxidase-antiperoxidase. Arrows point to mitotic figures. Final magnification, ×364.

gel electrophoresis, silver staining of gels, and Western blot (immunoblot) analyses were all done as described elsewhere (20).

Antibodies used for immunostaining of cell cultures or for Western blot analyses. Goat anti-rabbit immunoglobulin G was purchased from United States Biochemical Corp., Cleveland, Ohio. Rabbit antisera to glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), neuron-specific enolase (NSE), and fibronectin, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, peroxidase-antiperoxidase complex, and glucose oxidase–anti-glucose oxidase complex were all purchased from Accurate Chemical and Scientific Corp., Westbury, N.Y.

RESULTS

Differentiation of cells in MEB cell cultures. (i) Primary cultures. Young (6-day-old) primary MEB cell cultures displayed intense mitotic activity (Fig. 1A); at that stage, most of the large cells in the basal layer reacted diffusely with antisera to both GFAP and NSE, markers for identification of astrocytes and neurons. Subsequently, these cells progressively differentiated into mature astrocytes characterized by a very dense network of cytoplasmic processes reacting intensely and specifically with anti-GFAP (see Fig. 5A). In primary cultures defined as aged (i.e., 21 to 25 days old), such mature astrocytes were the dominant cell type. Among the cells lying on top of the astrocyte layer were anti-MBP-reactive oligodendrocytes (see Fig. 5B) and anti-NSE-positive neurons. At 3 weeks, most of the latter were rounded and formed clusters surrounded by massive arrays of fine processes; however, occasional morphologically typical neurons were present (see Fig. 5P). By 6 to 9 weeks, many cells had shapes typical of mature neurons (see Fig. 5C).

In addition to oligodendrocytes and neurons, the top layers of the cultures contained some very small cells with scant cytoplasm and very thin, often highly branched cytoplasmic processes; these cells failed to react with either anti-MBP or anti-GFAP, and we are not certain of their nature. Finally, aged MEB cultures contained variable numbers of cells reacting with anti-fibronectin, presumably fibroblasts; these were most numerous in dense areas of the cultures.

(ii) Secondary AE cultures. As described in Materials and Methods, MEB cultures destined to be subcultured under conditions yielding cell populations consisting of 90 to 99% astrocytes were initiated in serum-free DME supplemented with various growth factors recommended by Fischer et al. (6) and subcultured in the same medium after 2 weeks on poly-L-lysine-coated plastic or glass. At that stage, more than 99% of all cells have been reported to react with antibody to vimentin (a marker for immature astrocytes) but not with anti-GFAP (6). After 3 days, serum-free DME was replaced with DME containing 10% horse serum. Three to four days later, the great majority of cells and their processes began to react specifically with anti-GFAP (Fig. 2A and B). These cells were astrocytes clearly at an earlier stage of differentiation than were those in aged primary cultures, which were maintained from the beginning in serum-enriched medium (see Fig. 2C and D for comparison). In the experiments described below, the cultures were infected at this stage in order to prevent the remaining nonastroglial cells from outgrowing astrocytes after extended maintenance in serum-containing medium.

Growth curves for PR8, WS, and WSN in MDCK and MEB cell cultures. CEF, MDCK, and secondary astrocyte-enriched MEB cell cultures were infected with each virus at an MOI of 3 PFU(TR−+) per cell, and medium and cells were harvested separately at 1, 4, 7, 24, and 48 h postinfection (p.i.) (only at 1, 4, and 7 h p.i. for CEF). The peak titers of cell lysates at 7 h p.i., plotted in Fig. 3 as PFU(TR++) per 10^5 cells, were similar for all three viruses in CEF and MDCK cells. In AE cultures (ASTRO in Fig. 3), the titers of cell lysates were 10-fold lower after infection with PR8 than with WS or WSN. In all instances, cell-associated plaque titers decreased between 7 and 48 h p.i., indicating lack of secondary spread to new cells due to absent HA cleavage (WS and PR8), interferon production (unpublished preliminary findings by P. Husak in this laboratory), and/or virus-induced cytopathic effects (WSN).

In the media, PFU titers of all three viruses decreased from their peaks in MDCK cultures but underwent parallel, gradual increases between 7 and 48 h in AE cultures, which suggested a slow rate of continuing virion maturation and release. The fact that this phenomenon occurred equally with all three viruses indicates that (i) it was unaffected by the presence or absence of HA cleavage, (ii) the initially

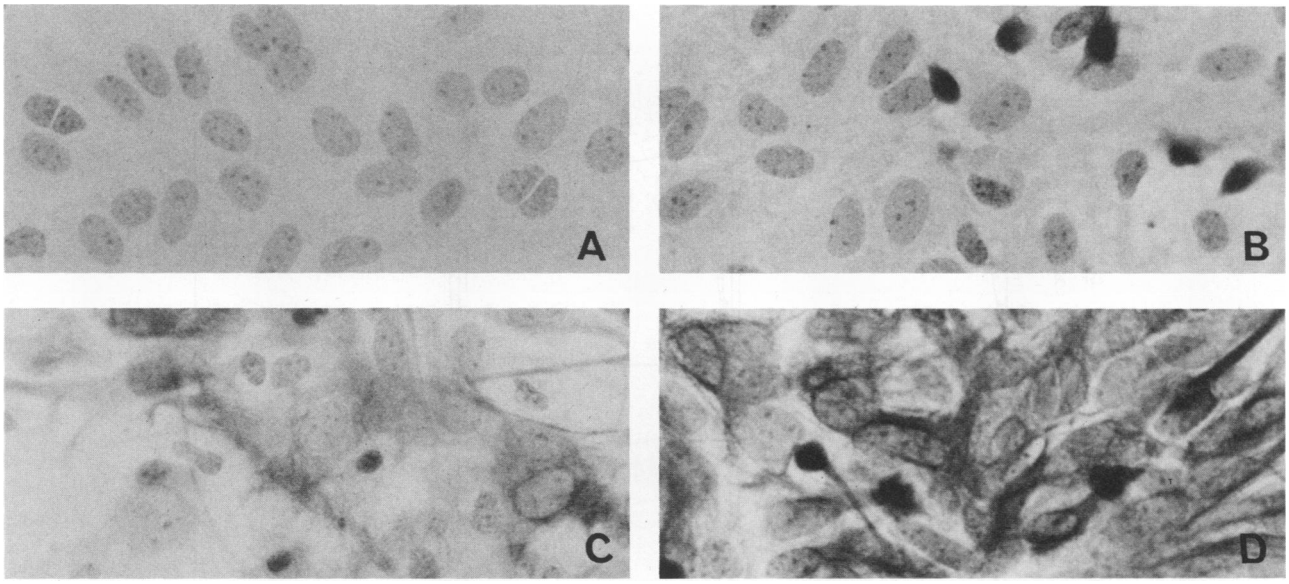

FIG. 2. Uninfected secondary AE (A and C) and uninfected 25-day-old primary MEB (B and D) cell cultures; immunoreactions with normal rabbit serum (A and B) and rabbit antiserum to GFAP (C and D), localized with peroxidase-antiperoxidase. Final magnification, ×475.

infected cells remained capable of supporting virion maturation, and (iii) some other factor was responsible for the reduced capacity of these cells to assemble and release virions during the initial 7 h but enabled this process to continue during the remainder of the experiment. We shall present evidence pointing to continuing slow synthesis of M1 protein as one such factor (G. L. Bradshaw and R. W. Schlesinger, manuscript in preparation).

Single-cycle yields of plaque-forming and HA particles in infected MEB cultures. To minimize complications resulting from multiple cycles of virus replication, such as accumulation of defective-interfering genomes or virions and effects of interferons, we infected cell cultures at relatively low MOI (usually 3 PFU per cell) and harvested media and cells for various assays at 7 to 9 h p.i. It should be noted that the input was always calculated from assays on MDCK cells with trypsin-supplemented overlay medium [PFU(TR−+)]. In contrast, the liquid medium under which experimental cell cultures were maintained did not contain trypsin. Under these conditions, the effective MOI of stock virus preparations, as judged by percentages of MDCK cells reacting with antibody to NP at 7 h p.i., has averaged 54, 63, and 59% for PR8, WS, and WSN, respectively (e.g., see Fig. 5E and G).

Figure 4 presents the results of several experiments, which can be summarized as follows. Yields of total cell-associated HA particles were equivalent in MDCK and CEF cultures, 2- to 3-fold lower in young MEB cultures, and approximately 10-fold lower in aged MEB or secondary AE cultures, all infected with any of the three viruses. Titers of cell-associated PFU(TR++) averaged 1% of the number of HA particles in MDCK, CEF, and young MEB cultures but were greatly reduced, absolutely as well as relative to number of HA particles, in aged MEB cultures, especially those infected with PR8. Secondary cultures, in which astrocytes, though enriched, were less mature than those in aged MEB cultures (see above), produced more infectious virions than did the latter. The percentage of HA particles and trypsin-activated PFU released into medium, relative to cell-associated titers, was comparable for all virus-cell combinations, except for higher rates in MDCK cells, where it probably reflects early cell damage induced by all three viruses (see below).

NA activity associated with HA particles. In view of the critical role assigned to WSN RNA segment 6 as contributing to neurovirulence, it was of interest to determine whether brain-derived cells were in some way deficient in synthesizing functional NA of WS or PR8 viruses in normal amounts. This was done by carrying out comparative kinetic assays of NA activity on HA materials derived from infected eggs or cell cultures, with fetuin as the substrate. In the experiments shown in Table 1, crude allantoic fluids and cell culture media were used as such. All cell-associated particles were concentrated by hemadsorption, elution, and pelleting as described above. Because of the low activity of WSN on fetuin as a substrate (reviewed in reference 25), all samples infected with this strain failed to give interpretable results. Within the limits of accuracy of the assay method, the average NA activity per HA particle (Table 1) was reasonably constant and specific for the two strains in the form of crude or purified allantoic fluid stock viruses and in media or cell extracts from MDCK and CEF as well as young, aged, and AE MEB cell cultures. The findings are similar to those reported by Rott and Schäfer (17) for standard or defective-interfering forms of avian or human strains and for cell-associated noninfectious HA particles defined as viromicrosomes (20).

Immunostaining of cell cultures with antibodies to viral nucleoprotein (NP) and M1 proteins. The restricted ability of primary aged MEB cultures to produce potentially (i.e., trypsin-activated) infectious PR8, WS, and even WSN virions suggested that there might be a correlation with cell-dependent or virus strain-specific restriction in the expression of M1 protein. Accordingly, infected cell cultures were examined by immunostaining with antibodies to viral NP and M1 protein, using methods and reagents described in Materials and Methods and in legends to tables and figures.

(i) MDCK cells. At 7 h after infection with either WSN (Fig. 5E and F) or PR8 (Fig. 5G and H), MDCK cells reacted identically, both in numbers and intensity, with anti-NP (Fig. 5E and G) or anti-M1 (Fig. 5F and H); all positive cells

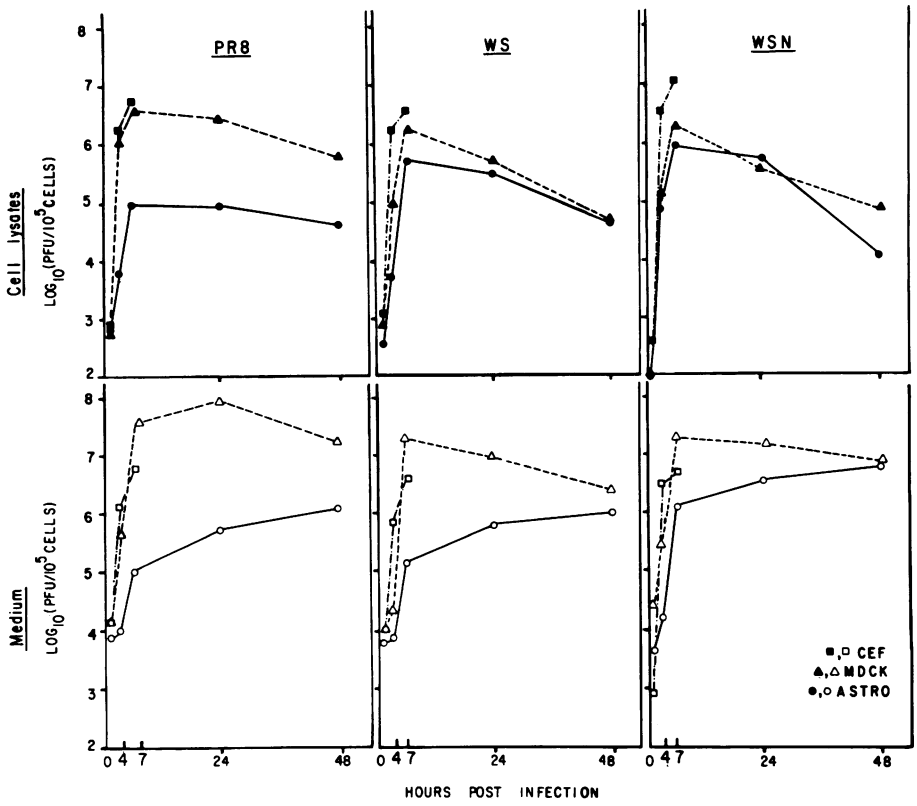

FIG. 3. Forty-eight hour growth curves for PR8, WS, and WSN (MOI = 3 PFU per cell) in CEF, MDCK, or secondary AE MEB cell cultures (ASTRO). At each time point, cell lysates and media were prepared as described in Materials and Methods. All points represent titers expressed as PFU(TR++) per 10^5 cells.

showed cytoplasmic staining and some intranuclear concentration of NP. Although no evidence for cytopathic effects was visible in these cells at 7 h, all such MDCK cell cultures underwent extensive degeneration after additional overnight incubation, regardless of the infecting virus strain (not shown).

(ii) Young (6-day-old) MEB cultures. Young cultures contained predominantly astrocyte precursor cells, which reacted diffusely with antibodies to GFAP as well as to NSE. Such cultures were almost as permissive as CEF or MDCK cells for all three viruses (Fig. 4). Not surprisingly, these cultures allowed full expression of M1 as well as NP reactivity; they retained the capacity to undergo mitosis after infection with PR8 (Fig. 1B and C).

(iii) Aged (25-day-old) MEB cultures. WSN-infected astrocytes showed strong cytoplasmic and nuclear reactions with anti-NP (Fig. 5I) and intense cytoplasmic reactions with anti-M1 (Fig. 5J); oligodendrocytes stained with equal intensity and distribution after reactions with both antibodies (Fig. 5I and J) and showed definite evidence of pycnotic degeneration. After infection with PR8, the response of astrocytes was characterized by intranuclear accumulation of NP (Fig. 5K), with greatly reduced or absent reaction with anti-M1 (Fig. 5L) (see also Fig. 5D, in which PR8-infected

TABLE 1. Neuraminidase activities in PR8- and WS-infected allantoic fluid and cell cultures

Material tested		Mean V_{max}[a] for sample infected with:	
Source	Type of sample[b]	PR8	WS
Allantoic fluid	Crude	138 (2) [126, 147]	49 (2) [45, 53]
	Purified stock	153 (5) [86–228]	53 (1)
MDCK cells	Cell-associated	211 (9) [126–348]	30 (5) [17–47]
	Medium	159 (1)	78 (1)
CEF	Cell-associated	192 (1)	65 (2) [55, 76]
	Medium	421 (2) [354, 488]	137 (2) [112, 163]
Young MEB	Cell-associated	434 (2) [415, 453]	ND
	Medium	282 (1)	ND
Aged MEB	Cell-associated	202 (2) [202, 202]	ND
AE	Cell-associated	188 (1)	35 (2) [28, 41]

[a] Expressed as 10^{-12} μg of *N*-acetylneuraminic acid released per HA particle per min, using fetuin as the substrate. Determinations were by direct measurements or from regression analyses of Lineweaver-Burk plots. For kinetic assays, the fetuin concentration ranged from 180 to 8,100 μg per tube. Figures in parentheses indicate number of assays; those in brackets indicate range of V_{max}. ND, No data available.

[b] See Materials and Methods. All cell cultures were harvested at 7 to 8 h p.i.

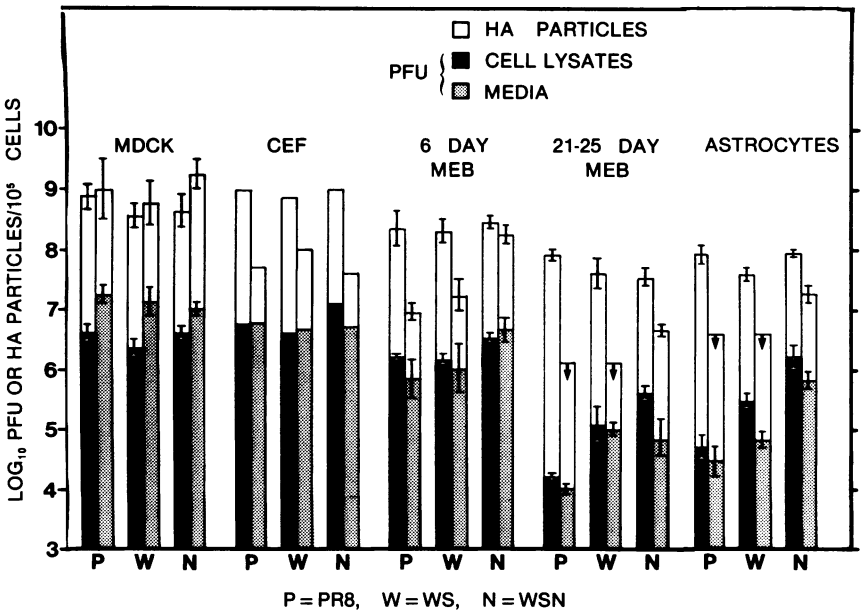

FIG. 4. Single-cycle (7 to 9 h p.i.) yields of HA particles and PFU(TR++) in cell cultures infected with PR8, WS, or WSN, all at an MOI of 3 PFU/cell. Astrocytes, secondary AE cultures. Double columns represent cell lysates (left) and media (right). All assays and calculations of numbers of HA particles are expressed per 10^5 cells. Vertical lines indicate standard deviations from the means. Values for CEF represent a single experiment. Arrows indicate lack of HA activity at lowest dilution tested.

astrocytes were doubly marked with anti-GFAP [blue] and anti-NP [brown]). In contrast, oligodendrocytes in the same cultures reacted strongly with both anti-NP (Fig. 5K) and anti-M1 (Fig. 5L) throughout the cytoplasm and cytoplasmic processes; neither astrocytes nor oligodendrocytes infected with PR8 showed cytopathic effects at 8 h p.i.

(iv) Mature neurons in older (9-week-old) MEB cultures. Neurons in older cultures infected with PR8 also differed from astrocytes in that they gave strong reactions with anti-NP (Fig. 5N) as well as anti-M1 (Fig. 5O), both evident throughout the cell body and processes. A dramatically strong reaction with anti-NP antibody is illustrated in a mature neuron encountered in an MEB culture infected at age 16 days with PR8 at an MOI of 0.1 PFU per cell and examined 72 h p.i. (Fig. 5P).

(v) Fibronectin-reactive cells. The fibronectin-reactive cells in any infected MEB culture examined have failed to react with either of the antiviral antibodies tested (not shown).

Table 2 summarizes anti-M1 and anti-NP reactivities of PR8-, WS-, and WSN-infected MDCK cells, as well as the mature astrocyte and nonastrocyte subpopulations of similarly infected aged MEB cultures, in terms of numbers of cells and relative staining intensities. PR8-infected astrocytes showed the greatest restriction of M1 expression, the least was found in WSN-infected cells, and intermediate degrees were found in WS-infected cells. In contrast, other cells in the same preparations, specifically those identifiable as oligodendrocytes or neurons, showed no such discrimination between the three virus strains and reacted very much as did MDCK cells.

Evidence for persistence of intranuclear NP in PR8-infected MEB cell cultures. Occasionally, we have examined PR8-infected secondary AE cultures for persistent nuclear immunostaining reactions with polyclonal anti-NP antibody. One such culture gave positive reactions in about 3% of the astrocytes at 3 weeks p.i., among them the single cell in anaphase shown in Fig. 5Q. Even at 8 weeks, intranuclear NP has been demonstrated in 0.07 to 0.2% of the cells. These observations, along with the demonstrated retention of mitotic activity in PR8-infected young MEB cultures shown in Fig. 1B and C, may be relevant to possible pathogenetic mechanisms. They are under further study, with use of mitotic arrest.

Immunostaining with antibody to NS1 protein. In limited experiments, infected MDCK and aged primary MEB cell

TABLE 2. Immunoreactivity of PR8-, WS-, and WSN-infected MDCK and aged MEB cultures 8 p.i. (MOI = 3 PFU per cell)

Cell type	Virus	Monoclonal antibody	No. stained[a]/ ×1,000 field	M1/NP	Relative staining intensity
MDCK	PR8	NP	41.1 + 4.0		++++
		M1	36.4 + 1.8	0.89	++++
	WS	NP	50.3 + 4.4		++++
		M1	44.5 + 5.2	0.88	++++
	WSN	NP	26.1 + 1.7		++++
		M1	21.9 + 2.4	0.84	++++
ODC,[b] neurons, and others excluding astrocytes	PR8	NP	12.4 + 1.8		++++
		M1	11.9 + 1.9	0.96	++++
	WS	NP	14.7 + 2.1		++++
		M1	17.4 + 2.4	1.18	++++
	WSN	NP	14.1 + 2.4		++++
		M1	15.5 + 2.5	1.10	++++
Astrocytes	PR8	NP	19.9 + 1.2		++++
		M1	3.2 + 0.4	0.16	+
	WS	NP	49.8 + 3.1		++++
		M1	18.1 + 1.9	0.36	++
	WSN	NP	23.5 + 2.5		++++
		M1	19.7 + 2.5	0.84	+++

[a] 10 to 20 fields counted per antibody per experiment. Aged MEB values represent pooled data from two experiments. MDCK values are from one experiment.

[b] ODC, Oligodendrocytes.

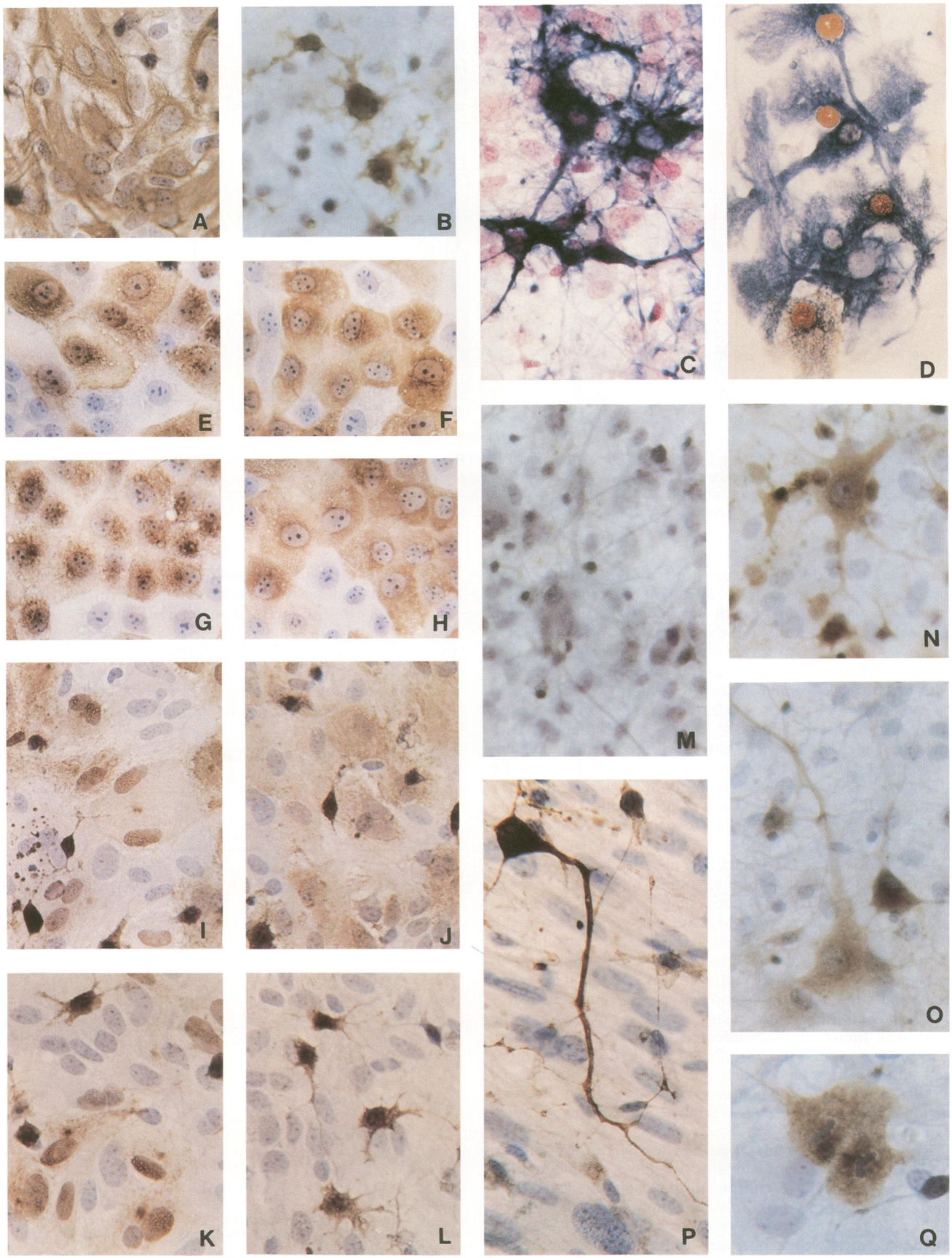

FIG. 5. Immunostaining of cell cultures with antibodies to cellular or viral proteins (see legend on facing page). Average magnification, ×364.

cultures were immunostained after reaction with polyclonal rabbit antiserum to NS1 protein, kindly supplied by M. Krystal and P. Palese. At 7 h p.i., intracellular compartmentalization of NS1 was generally similar to that of NP, i.e., mainly intranuclear in mature astrocytes and mainly cytoplasmic in other MEB cell types and in MDCK cells. Although the intensity of NS1 reactivity was comparable for all three viruses in MDCK cells, it was less in astrocytes infected with PR8 than in those infected with WS or WSN; in contrast, in the other cell types present in aged MEB cell cultures, PR8 induced a more intense anti-NS1 PAP staining reaction than did either WS or WSN (not shown). More definitive corroborative evidence concerning the expression of gene segment 8, based on radiochemical analysis of viral proteins, will be reported in a forthcoming article (Bradshaw and Schlesinger, in preparation).

Quantitation of NP and M1 protein. Protein quantitation by Western blot analysis of 10% sodium dodecyl sulfate-polyacrylamide gel electropherograms was carried out as described elsewhere (20). Two examples are shown in Fig. 6. Figure 6A compares extracts of young and aged primary MEB cultures prepared 8 h after infection with one of the three virus strains at an MOI of 3 PFU per cell and each extract run undiluted (20 μg of total protein; lanes a) and diluted 1:2 (10 μg of total protein; lanes b). It is evident that there was a marked reduction of M1 protein, relative to NP, in the 21-day-old cultures infected with either virus compared with the corresponding 6-day-old cultures. In Fig. 6B, similar comparisons are shown between MDCK cells and secondary AE MEB cultures but harvested at 1 (lanes a) and 8 (lanes b) h p.i. In accordance with the relatively early stage of differentiation and less restricted permissivity of these astrocytes for all three viruses (Fig. 4), the expression levels of M1 protein at 8 h p.i. (lanes b) were strikingly higher than in the 21-day-old primary MEB cultures.

LEGEND TO FIG. 5.

Figure 5 panel	Cell culture	Virus	MOI (PFU/cell)	Time p.i.	Antibody to[a]	Localizing stain[b]
A	25-day MEB	None			GFAP (p)	PAP
B	22-day MEB	None			MBP (p)	PAP
C	9-wk MEB	None			NSE (p)	GAG
D	5-wk AE	PR8	3	6 h	NP (m)	PAP
					GFAP (p)	GAG
E	MDCK	WSN	3	7 h	NP (m)	PAP
F	MDCK	WSN	3	7 h	M1 (m)	PAP
G	MDCK	PR8	3	7 h	NP (m)	PAP
H	MDCK	PR8	3	7 h	M1 (m)	PAP
I	25-day MEB	WSN	3	8 h	NP (m)	PAP
J	25-day MEB	WSN	3	8 h	M1 (m)	PAP
K	25-day MEB	PR8	3	8 h	NP (m)	PAP
L	25-day MEB	PR8	3	8 h	M1 (m)	PAP
M	9-wk MEB	None			NP (m)	PAP
N	9-wk MEB	PR8	3	7 h	NP (m)	PAP
O	9-wk MEB	PR8	3	7 h	M1 (m)	PAP
P	16-day MEB	PR8	0.1	72 h	NP (p)	PAP
Q	31-day AE	PR8	3	3 wk	NP (p)	PAP

[a] In panels D, E, G, I K, M, and N, anti-NP HT-103 ascitic fluid, from P. Palese; in panels F, H, J, L, and O, anti-M1 289/2 acitic fluid, from K. van Wyke-Coelingh; in panels P and Q, anti-NP as in Fig. 1. p, Polyclonal; m, monoclonal.

[b] PAP, Peroxidase-antiperoxidase; GAG, (glucose oxidase–anti-glucose oxidase).

DISCUSSION

We conclude from these studies that maturation of astrocytes in in vitro MEB cell cultures is associated with decreased ability of such cultures to support at least one cycle of complete replication of the non-neurotropic PR8 and WS strains of type A influenza virus (H1N1 subtype) and even of the neurovirulent WSN variant, as measured by the endpoint of fully assembled progeny virions that register as trypsin-activated plaque formers (Fig. 4).

Mature astrocytes are the predominant cell type in aged (≥21-day-old) MEB cultures, in which the single-cycle yields of such virions are reduced 100- to 1,000-fold with PR8 compared with those from similarly infected MDCK cells or CEF (reduced less with WS and least with WSN). Yield reductions are less pronounced for PR8 and WS and insignificant for WSN in secondary MEB cultures in which 90 to 99% of all cells are astrocytes just beginning to differentiate. In contrast, young (i.e., 6-day-old), undifferentiated MEB cultures produce comparable PFU yields of all three viruses that are only two- to threefold lower than those in CEF cultures.

Compared with these differences in infectious titers, the total number of HA particles recovered from cell lysates by hemadsorption and elution are characteristic of each type of cell culture, regardless of the infecting virus strain. In general, the percentages of cell-associated HA particles registering as trypsin-activated PFU are very low, a finding consistent with the view that most of them are precursor forms (viromicrosomes) derived from cytoplasmic membranes, as discussed elsewhere (20). In the media, the PFU/HA ratios tend to be higher, as expected of spontaneously released virions. Exceptions are WSN-infected MEB cultures and MDCK cells infected with any of the three strains. These all develop cytopathic effects and probably release precursor particles along with mature virions even before damage is visible in the light microscope.

Restricted assembly of progeny virions is correlated with restricted expression of M1 protein (relative to NP) in mature astrocytes, as demonstrated by immunostaining. No similar cell-dependent and virus strain-specific restrictions are seen in oligodendrocytes or neurons; both produce M1 protein and NP in cell bodies and processes with the same frequency and with the same immunostaining intensity as do MDCK cells, regardless of which strain is used to infect them. More limited experiments involving immunostaining with a polyclonal antiserum to NS1 protein reveal that that protein is reduced only in PR8-infected, not in WS- or WSN-infected, mature astrocytes. Interestingly, oligodendrocytes and neurons infected with PR8 react more intensely with this antibody than do sister cultures infected with the other two strains.

Taken in conjunction with the apparent specificity of the NA of WSN in facilitating cleavage of HA (16, 21, 22, 26), the differences in responses in terms of M1 and NS1 protein expression are compatible with the report that, among reassortants derived from crosses of WSN with a non-neurovirulent H3N2 strain, only those carrying WSN gene segments 6, 7, and 8 can undergo multiple cycles of replication in mouse brain or neuroblastoma cell cultures and express maximal acute neurovirulence in mice after i.c. inoculation (26). The specific gene or combination of genes responsible for the latter, as distinct from indirect promotion of HA cleavage, remains to be identified.

It is important to reemphasize that the implication of RNA segments 6, 7, and 8 in neurovirulence is based solely on

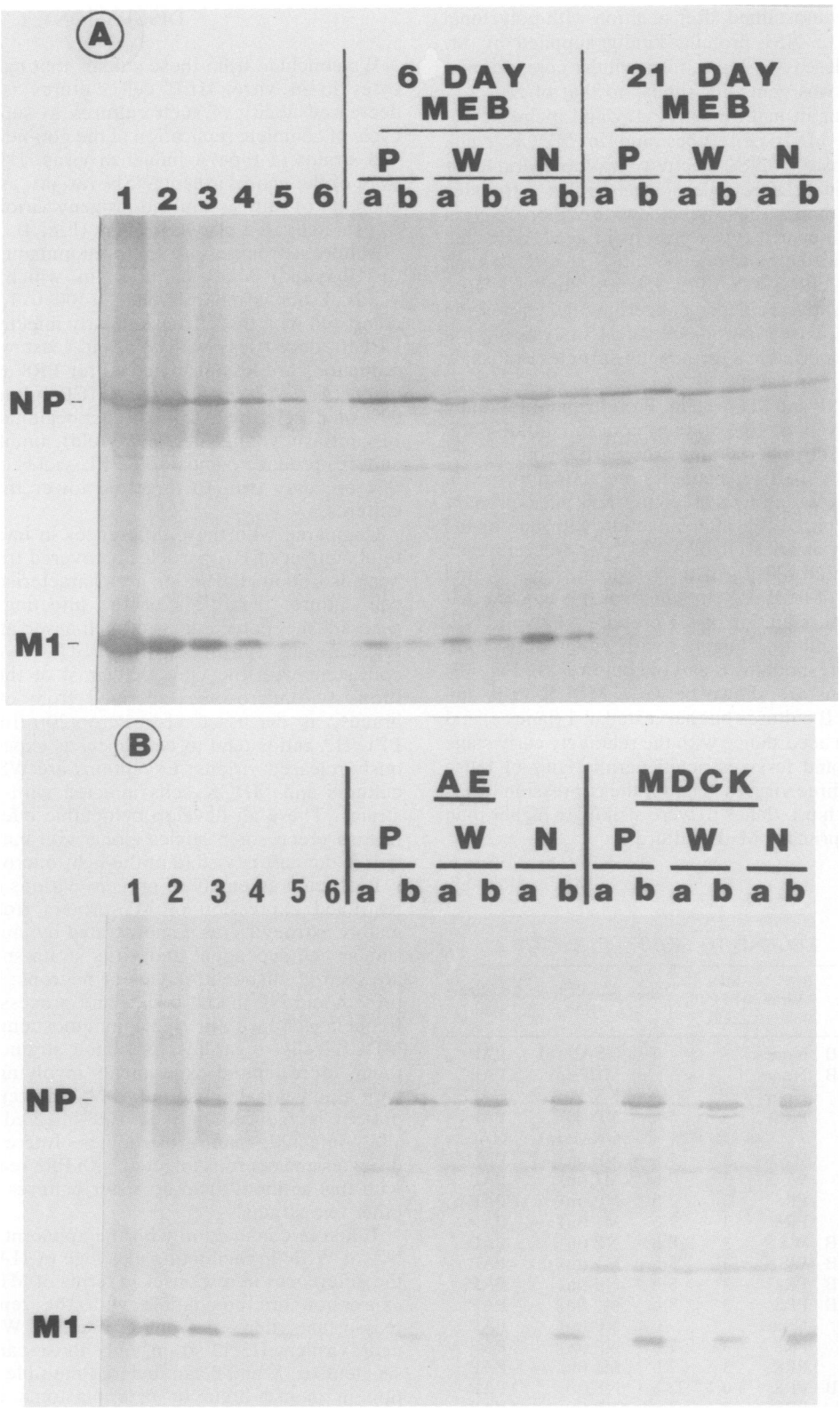

FIG. 6. Western blots of infected cell lysates separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Shown are double reactions with polyclonal anti-NP and anti-M1 antibodies (same as in Fig. 1), localized with peroxidase-antiperoxidase. P, PR8; W, WS; N, WSN. (A) Primary 6- and 21-day-old MEB cultures, all collected at 8 h p.i. Lanes: 1 to 6, fourfold serial dilutions of purified PR8 allantoic fluid stock virus (12 to 0.012 μg of total protein per lane); a and b, 20 (a) and 10 (b) μg of total protein of each lysate. (B) MDCK cells and secondary AE MEB cultures. Lanes: 1 to 6, as in panel A but, with fivefold serial dilutions (10 to 0.0032 μg of total protein per lane); a and b, 20 μg of total protein per lane of cell lysates prepared at 1 (a) and 8 (b) h p.i.

analysis of reassortants of WSN with a single H3N2 strain, A/Aichi/2/68 (16, 26). Compared with the latter strain, PR8 and especially WS are close relatives of WSN. Therefore, induction by these two strains of seemingly normal amounts of immunoreactive M1 and NS1 proteins (relative to NP) in oligodendrocytes and neurons suggests that perhaps all or most of the fully assembled virions produced in aged MEB cultures and released into the medium originate in these cells, not in astrocytes.

We believe that aged MEB cultures offer a model with analogies to brains of mice inoculated i.c. with these two non-neurovirulent strains. In the latter, M1 protein could not be detected in sections or in Western blots even though it was detectable in particles purified and concentrated from such brains by hemadsorption, elution, and pelleting. Western blot analyses showed that ratios of peroxidase-antiperoxidase staining intensities with antibodies to M1 and NP were comparable for particles from brains infected with all three viruses (20).

It seems reasonable to suggest that the toxic neurological signs and acute deaths observed in mice between 12 and 72 h after i.c. inoculation with large amounts of PR8, WS, and other non-neurotropic H1N1 strains (8, 9, 15) may be related to productive involvement of neurons and oligodendrocytes similar to that observed in our MEB cell cultures. Differences between specialized nerve cell types with regard to the ability to support virus replication or persistence, to undergo acute or delayed cytopathic changes, or to induce progressive demyelinating and other degenerative neuropathology have been reported for several neurotropic viruses or mutants. Of special relevance to the findings reported here are those concerned with restricted expression of M proteins in brains or nerve cell cultures infected with measles or vesicular stomatitis viruses (extensively reviewed in references 5 and 11 through 13) and with the generalization that viral persistence is more often characteristic of astrocytes than of neurons, oligodendrocytes, Schwann cells, and ependymal and meningeal cells, all of which seem to be more likely to support acute productive infections (5, 11). These related observations indicate that, as would be expected, virus-specific and cell-dependent genetically determined functions cooperate in all these situations.

This article has defined the systems that we have developed to investigate these relationships. In subsequent reports, we shall describe quantitative analyses of radioactively labeled viral proteins (Bradshaw and Schlesinger, in preparation) and initial studies of the roles played by transcriptional or posttranscriptional regulation in the virus-cell systems described here (G. L. Bradshaw, C. D. Schwartz, P. J. Husak, and R. W. Schlesinger, submitted for publication).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-21562 from the National Institute of Allergy and Infectious Diseases, and by Public Health Service institutional national research service award CA-09069 from the National Cancer Institute.

We are grateful to D. Bucher, P. Palese, M. Krystal, K. van Wyke-Coelingh, and R. A. Lamb for their generosity in making available the antibodies to specific viral proteins identified in Results.

LITERATURE CITED

1. **Aminoff, D.** 1961. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **81**:384–392.
2. **Baylor, N. W., Y. Li, Z. Ye, and R. R. Wagner.** 1988. Transient expression and sequence of the matrix (M1) gene of WSN influenza A virus in a vaccinia vector. Virology **163**:618–621.
3. **Bradshaw, G. L., and R. W. Schlesinger.** 1987. Quantitation of viral progeny and proteins in mouse brain cells infected with WSN or non-neurovirulent H1N1 influenza strains, p. 349–356. *In* B. W. J. Mahy and D. Kolakofski (ed.), The biology of negative strand viruses. Elsevier Science Publishers, Amsterdam.
4. **Clark, C. A., E. C. Downs, and F. J. Primus.** 1982. An unlabeled antibody method using glucose oxidase-antiglucose oxidase complexes (GAG): a sensitive alternative to immunoperoxidase for the detection of tissue antigens. J. Histochem. Cytochem. **30**:27–34.
5. **Dubois-Dalcq, M., B. Rentier, E. Hooghe-Peters, M. V. Haspel, R. L. Knobler, and K. Holmes.** 1982. Acute and persistent viral infections of differentiated nerve cells. Rev. Infect. Dis. **4**: 999–1014.
6. **Fischer, G., A. Leutz, and M. Schachner.** 1982. Cultivation of immature astrocytes of mouse cerebellum in a serum-free hormonally defined medium. Appearance of the mature astrocytic phenotype after addition of serum. Neurosci. Lett. **29**:297–302.
7. **Francis, T., and A. E. Moore.** 1940. A study of the neurotropic tendency in strains of the virus of epidemic influenza. J. Exp. Med. **72**:717–728.
8. **Henle, G., and W. Henle.** 1944. Neurological signs in mice following intracerebral inoculation of influenza viruses. Science **100**:410–411.
9. **Henle, G., and W. Henle.** 1946. Studies on the toxicity of influenza viruses. I. The effect of intracerebral injection of influenza viruses. J. Exp. Med. **84**:623–637.
10. **Honegger, P., and E. Richelson.** 1976. Biochemical differentiation of mechanically dissociated mammalian brain in aggregating cell culture. Brain Res. **109**:335–354.
11. **Johnson, R. T.** 1982. Viral infections of the nervous system, p. 1–433. Raven Press, New York.
12. **Koolen, M. J. M., and M. J. Buchmeier.** 1986. Experimental models of virus attentuation and demyelinating disease. Microbiol. Sci. **3**:68–72.
13. **Kristensson, K., and E. Norrby.** 1986. Persistence of RNA viruses in the central nervous system. Annu. Rev. Microbiol. **40**:159–184.
14. **Lamb, R. A.** 1983. The influenza virus RNA segments and their encoded proteins, p. 21–69. *In* P. Palese and D. W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, New York.
15. **Mims, C. A.** 1960. An analysis of the toxicity for mice of influenza virus. I. Intracerebral toxicity. Br. J. Exp. Pathol. **41**:586–592.
16. **Nakajima, S., and A. Sugiura.** 1980. Neurovirulence of influenza virus in mice. II. Mechanism of virulence as studied in a neuroblastoma cell line. Virology **101**:450–457.
17. **Rott, R., and W. Schäfer.** 1961. Untersuchungen über die hämagglutinierenden-nichtinfektiösen Teilchen der influenzaviren. II. Mitt.: Vergleichende Untersuchungen über die physikalisch-chemischen und biologischen Eigenschaften der Teilchen. Z. Naturforsch. **16b**:310–321.
18. **Schlesinger, R. W.** 1950. Incomplete growth cycle of influenza virus in mouse brain. Proc. Soc. Exp. Biol. Med. **74**:541–548.
19. **Schlesinger, R. W.** 1953. The relation of functionally deficient forms of influenza virus to viral development. Cold Spring Harbor Symp. Quant. Biol. **18**:55–59.
20. **Schlesinger, R. W., G. L. Bradshaw, F. Barbone, M. Reinacher, R. Rott, and P. Husak.** 1989. 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. J. Virol. **63**: 1695–1703.
21. **Schulman, J. L.** 1983. Virus-determined differences in the pathogenesis of influenza virus infection, p. 305–320. *In* P. Palese and D. W. Kingsbury (ed.), Genetics of influenza viruses. Springer-Verlag, New York.
22. **Schulman, J. L., and P. Palese.** 1977. Virulence factors of influenza A viruses: WSN virus neuraminidase required for

plaque production in MDBK cells. J. Virol. **24**:170–176.
23. **Smith, W., C. H. Andrewes, and P. P. Laidlaw.** 1933. A virus obtained from influenza patients. Lancet **i**:66–68.
24. **Stuart-Harris, C. H.** 1939. A neurotropic strain of human influenza virus. Lancet **i**:497–499.
25. **Sugiura, A.** 1975. Influenza virus genetics, p. 171–213. *In* E. D. Kilbourne (ed.), The influenza viruses and influenza. Academic Press, Inc., New York.
26. **Sugiura, A., and M. Ueda.** 1980. Neurovirulence of influenza virus in mice. I. Neurovirulence of recombinants between virulent and avirulent virus strains. Virology **101**:440–449.
27. **Werner, G. H., and R. W. Schlesinger.** 1954. Morphological and quantitative comparisons between infectious and non-infectious forms of influenza virus. J. Exp. Med. **100**:203–216.