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Down-Regulation of Glial Fibrillary Acidic Protein Expression during Acute Lytic Varicella-Zoster Virus Infection of Cultured Human Astrocytes

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The effects of the varicella-zoster virus (VZV) OKA vaccine strain in producing morphologic and antigenic changes in dissociated cultures of human fetal brain was investigated. Cultures containing 80% glial acidic fibrillary protein (GFAP), $GFAP^+$ (positive) astrocytes and 20% $GFAP^-$ (negative) fibroblastic-like cells were infected with cell-free VZV OKA at a multiplicity of infection of 0.1 plaque-forming units per cell. Cytopathic effects and significant viral antigen labeling with antibodies against VZV gpI and immediate-early (IE) protein 62 were first detected 6 to 7 days postinfection. Several observations indicated that astrocyte GFAP expression was altered and diminished as a result of VZV infection itself, thereby raising doubts about the utility of combining cell markers and viral antigenic labeling in assessing the susceptibility of neural cell types to viral infection. The down-regulation of GFAP expression by VZV appears to be mediated by early rather than late events in the viral replication cycle and may not be the result of virally induced global shut-off of host cell protein synthesis. Similar observations were made using VZV Ellen, a multipassaged, nonvaccine strain. These observations have potential *in vivo* implications related to histologic analysis of VZV-infected tissues and disease pathogenesis.

Varicella-zoster virus (VZV) is an important and frequent cause of disease in humans. During the primary infection of varicella (chickenpox), the virus is taken up in sensory nerve endings and then remains in latent form in the trigeminal and dorsal root ganglia (*1*). In the presence of various triggering factors, VZV may undergo reactivation, producing zoster (shingles). Both of these conditions may lead to various neurologic complications — such as encephalitis, myelitis, and arteritis — that are often serious and sometimes fatal (*2*). The pathogenesis of varicella, zoster, and their neurologic complications, however, is poorly understood. Much more is known about herpes simplex virus (HSV) latency and neurotropism than that of VZV, largely because VZV is highly cell associated, difficult to grow to high titers, and animal models of VZV infections are not well developed (*1, 2*). *In vitro* studies of the interaction of VZV with neural tissues have been very limited, although a previous detailed study from our laboratories examined the cell specificities of a wild-type, cell-free VZV strain in cultures of human fetal neural tissues (*3*). Other studies used cell-associated rather than cell-free VZV in neural cultures (*4, 5*).

A live attenuated VZV vaccine (OKA strain) is available in several countries for clinical use. It is of particular value in immunocompromised individuals in whom complications of VZV infections are more frequent than in normal persons (*6*). That the vaccine virus is capable of establishing latency is clear because zoster has occurred as a result of its reactivation. It is, therefore, important to acquire as much information as possible about this vaccine strain regarding its potential neurotropism. Accordingly, we initiated the current study of the effects of the VZV OKA strain in cultured human astrocytes using cell-specific markers to define unambiguously the virus–astrocyte interaction because of the critical functional significance of this cell type in the CNS. For example, there is evidence for the astrocyte's role in formation of the blood–brain barrier (*7*) and myelin (*8*), secretion of various cytokines (*9*) and glial growth factors (*10*), antigen presentation *in vitro* (*11*), and metabolic interaction with other neural cell types (*9*). We considered that VZV-induced alterations of astrocytic properties *in vitro* could be relevant to the pathogenesis of human neurologic disease. In this study, the expression of the glial fibrillary acidic protein (GFAP) was examined, since it is associated with glial intermediate filaments and is a widely used, astrocyte-specific marker (*12, 13*).

Human fetal brain tissue of 9-week gestational age was mechanically disrupted by aspiration through No. 19 gauge hypodermic needles, washed in Eagle's minimum essential medium (E-MEM), and placed into culture flasks pretreated with poly-D-lysine (0.1 mg/ml in distilled water). All dissociated cultures were established from individual brain specimens. Cultures were grown in E-MEM supplemented with 10% fetal bovine serum and

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gentamicin (25 μg/ml) and refed every 4 days. These cultures produced a heterogeneous population of astrocytes, neurons, and precursor oligodendrocytes. Cultures were passaged using 0.025% trypsin in 0.005% EDTA. For VZV infectivity and antibody staining reactions, cells were plated onto 13-mm glass coverslips in Linbro multiwell plates at a density of $2-4 \times 10^3$ cells per coverslip. Cultures were studied at passage 3, by which time only astrocytes and a few fibroblasts were present.

Cell-free VZV OKA strain prepared in MRC-5 cells was a gift from Dr. J. White (Merck Co., West Point, PA). The virus is lyophilized in concentrations of approximately 2×10^3 plaque-forming units (PFU) per vial. The OKA strain differs from American wild-type VZV isolates in that it has an additional *Bgl*I site lying within the *Bam*HI "D" fragment on restriction enzyme analysis (*14*). The extra restriction site is also present in the parental virus DNA and so is not produced by the attenuation process itself (*14*). Cell-free VZV Ellen strain (a multipassaged strain) was prepared in Mewo cells (obtained from Dr. C. Grose) as follows. Confluent monolayers of Mewo cells grown in E-MEM with 10% fetal calf serum supplemented with 2 m*M* L-glutamine, 26.3 mg/ml aureomycin, 25 mg/ml streptomycin, and 15.8 mg/ml penicillin (Quality Biological, Inc., Gaithersburg, MD) were infected at a 1:10 ratio with cell-associated VZV Ellen. After 2 days, monolayers showed 80 to 100% cytopathic effect (cpe). These infected monolayers were scraped in growth media and pelleted at 4° at 300 *g*. The infected cells were resuspended in sonication media (Dulbecco's PBS, 5% sucrose, 0.1% sodium glutamate, with 10 mg/ml human serum albumin) and sonicated on ice with a Heat Systems sonicator Model W220 (Farmingdale, NY) at setting 2 for 30 sec. Cell-free virus was separated from cell debris by centrifugation at 1000 *g* for 20 min at 4° and stored at −80° prior to use. Human fetal neural cells growing on glass coverslips were infected with VZV OKA or Ellen at a multiplicity of infection of 0.1 PFU/cell. After absorption for 1.5 hr at 37°, the virus inoculum was removed, and the cells were washed with medium and refed with fresh Dulbecco's MEM containing 10% fetal bovine serum. A total of eight independent infections was carried out to produce the data reported in this study. The VZV OKA strain was studied on all eight occasions, and VZV Ellen twice. Uninfected control cultures were included in all experiments.

Indirect immunofluorescence on cell cultures was performed as previously described (*15*) at 3 and 7 days after virus infection. Astrocytes were identified using a polyvalent rabbit antibody to GFAP (Dako, diluted 1:100). Mouse monoclonal anti-neurofilament antibody 1217 (diluted 1:500) was obtained from Affiniti, Inc. Mouse monoclonal antibody to the VZV gpI protein (diluted 1:1000) was obtained from Dr. B. Forghani, and mouse mAb to the VZV immediate-early (IE) protein of gene 62 (diluted 1:1000) was obtained from Chemicon Corp. (Temecula, CA). Mouse mAb to human HLA-DR (diluted 1:30) was obtained from Becton–Dickinson Reagents (San Jose, CA). Antibody binding was visualized with swine anti-rabbit IgG conjugated to rhodamine (Dako, diluted 1:40) or goat anti-mouse IgG conjugated to fluorescein (Dako, diluted 1:50). Prior to labeling, cultures were fixed in absolute methanol at −20° for 15 min. They were then washed in PBS and incubated with the primary antibodies for 30 min at room temperature, washed again in PBS, and then incubated with the appropriate fluorescent-conjugated antibodies for 30 min. In most experiments, double-labeling was employed, e.g., cultures were first exposed to a mixture of anti-GFAP and anti-gpI antibodies, followed after washing by a mixture of the two rhodamine and fluorescein-conjugated antibodies. After washing, the cells were mounted in glycerol/PBS onto glass slides and examined under a Zeiss ICM405 microscope equipped with epifluorescence. Cell numbers were assessed on duplicate slides semiquantitatively as previously described (*15*) by counting 150 cells in five separate fields of view, and the degree and type of cpe and immunofluorescent labeling were determined. The cpe consisted of loss of normal cell morphology, with rounding and clumping of cells.

Uninfected cultures comprised 70–80% $GFAP^+$ astrocytes (Fig. 1) and 20–30% $GFAP^-$ cells with fibroblastic morphology. At this time, no neurofilament$^+$ neurons were seen, since these were lost during serial passage. In both OKA- and Ellen-infected cultures at the early time point (3 days postinfection), no cpe was seen, and only a few areas of weak VZV gpI labeling were detected. In some experiments, labeling with antibody to immediate-early 62 protein was sought but, again, only a few cells were labeled. Labeling with gpI antibody was generally granular and cytoplasmic, but sometimes appeared membranous. When present, the anti-62 protein labeling was granular and nuclear/perinuclear in location. No cells doubly labeled for GFAP and gpI were seen at this stage.

At 7 days postinfection with both VZV OKA and Ellen, a few areas of focal cpe — sometimes forming small plaques — were observed. At this stage, based on five key experiments with VZV OKA, the average proportion of $GFAP^+$ cells in infected cultures was 43% (range 30–50%), compared with an average of 69% (range 50–80%) $GFAP^+$ cells in uninfected cultures. This difference was statistically significant (Table 1). There was no obvious, marked loss of total cells on the coverslips in infected compared with uninfected cultures. In infected cultures, $GFAP^+gpI^+$ cells constituted approximately 20%, and gpI^+ cells (irrespective of GFAP expression) constituted approximately 40% of the total cell population. No labeling with either anti-gpI or anti-IE 62 was detected in uninfected cultures.

At this later stage, at least four patterns of GFAP and gpI staining were seen. These were as follows: (*i*)

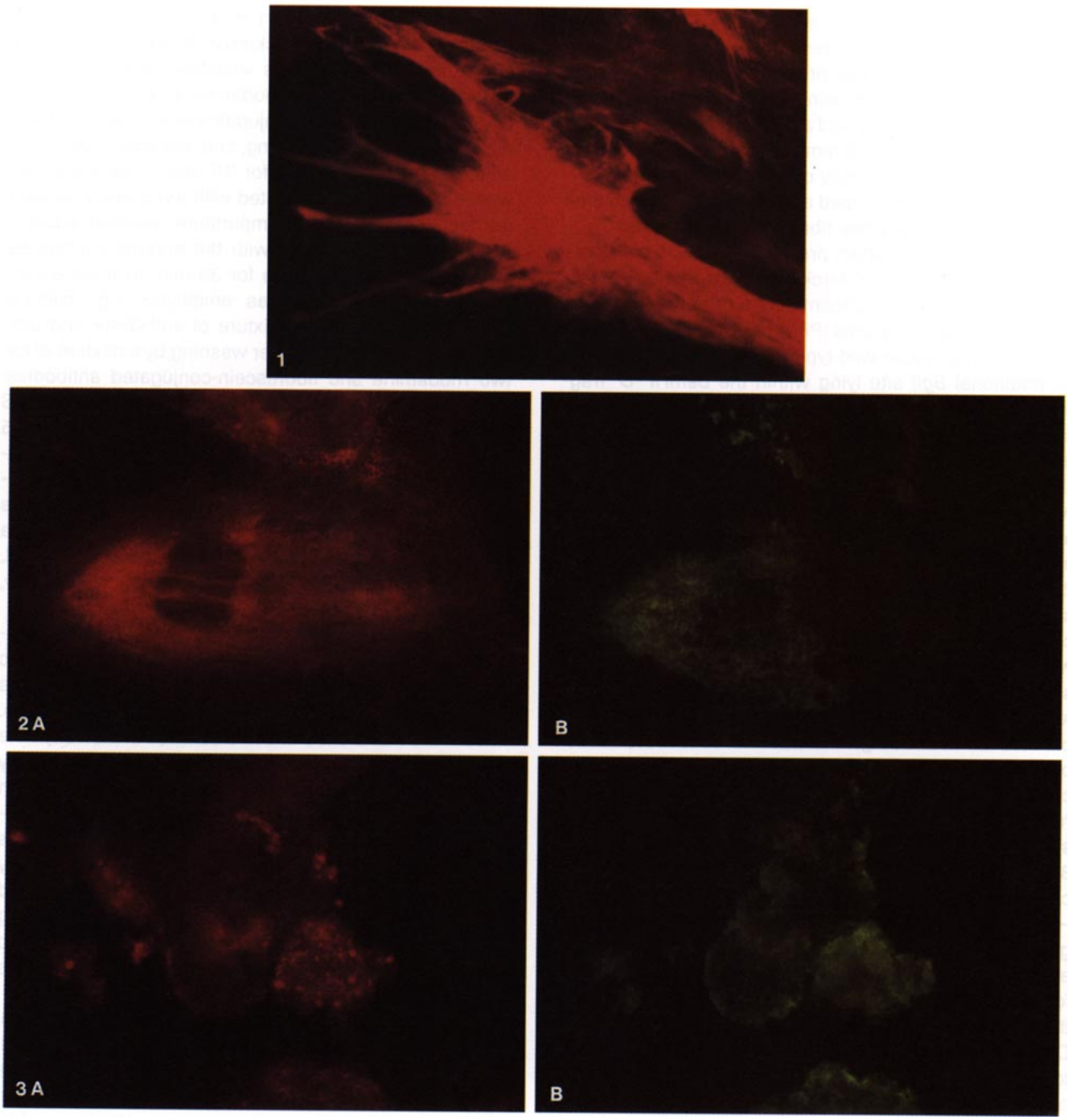

FIG. 1. GFAP$^+$ astrocyte in human brain culture. The cytoplasmic fibrillar staining pattern is evident using rhodamine-conjugated antibody and epifluorescence. Magnification ×475.

FIG. 2. GFAP$^+$gpl$^+$ astrocytes in human brain culture 7 days after infection with VZV OKA. (A) Two GFAP$^+$ cells are seen under rhodamine (red) fluorescence; (B) both of these cells are also seen to be double-labeled with antibody to gpl using fluorescein (green) fluorescence. Only part of the lower cell is double-labeled. In this experiment, GFAP$^+$ cells composed about 30% of the total population compared with about 70% in uninfected control cultures. Magnification ×475.

FIG. 3. GFAP$^+$gpl$^+$ cells showing a cpe in human brain cultures 7 days after infection with VZV OKA (same experiment as that shown in Fig. 2). (A) Several cells showing a rounded cpe are GFAP$^+$ seen under rhodamine fluorescence, but in a granular particulate staining pattern; (B) the same field viewed under fluorescein fluorescence shows that these cells are also strongly labeled with antibody to gpl. Magnification ×475.

GFAP$^+$gpl$^-$ cells where the GFAP staining was always very strong. Glial filaments were clearly seen in such cells, which composed approximately 10% of the total population; (ii) GFAP$^-$gpl$^+$ fibroblastic cells which composed approximately 20% of the total population; (iii) GFAP$^+$gpl$^+$ astrocytes, which composed approximately

TABLE 1

GFAP EXPRESSION IN INFECTED AND UNINFECTED HUMAN NEURAL CULTURES[a]

	Percentage GFAP positive
VZV infected	43 ± 8.4
Uninfected	69 ± 8.2

[a] GFAP expression was determined by counting flourescent positives in 150 cells in random fields on duplicate coverslips. The percentage GFAP positive cells averaged from five experiments with standard deviations is shown. Significantly fewer infected cells than uninfected cells expressed GFAP (two-tailed Student t test, $P < 0.05$).

10% of the population (Fig. 2). These cells were generally weakly labeled with both antibodies, and a small proportion of these (~25%) showed slight but definite loss of filamentous structure; and (iv) $GFAP^+gpI^+$ labeling was also seen in a pattern completely different from that of iii in approximately 5–10% of the total cells where such cells undergoing a cpe showed a particulate pattern of GFAP staining (contrasting with the usual diffuse cytoplasmic GFAP staining) together with often strong gpI staining (Fig. 3). Such cells were generally located in the vicinity of areas of focal cpe and were occasionally attached to cells within these areas.

The clear conclusion from all of these findings at 7 days postinfection was that GFAP expression in astrocytes was being down-regulated or modified in some way during the course of lytic VZV infection of these cultures. Both the absolute changes in $GFAP^+$ cell numbers compared with the uninfected control cultures and the gradation and pattern of GFAP and gpI coexpression provide strong evidence for this interpretation. While this may reflect diminished synthesis of GFAP during infection, the staining patterns in iii and iv also suggest that actual disassembly and/or destruction of the glial filaments may have been caused by the viral infection.

One possible mechanism of down-regulation of GFAP during VZV infection was through a general virus-induced shut-off of host cell protein synthesis. We therefore looked for possible down-regulation of other host cell proteins, namely class II MHC antigens, which have been shown *in vivo* to be up-regulated within the inflammatory lesions in VZV-associated encephalitis (*16*). It was found that at both 3 and 7 days after infection, approximately 3–5% of cells in both infected and uninfected cultures were labeled with an antibody against human HLA-DR antigens in a granular pattern of surface labeling (cells were labeled live in this experiment). Approximately 50% of these cells were $GFAP^+MHC\ DR^+$. There was no evidence of diminished class II antigen expression in the infected cultures and, presumably, the tissue culture procedure itself had in some way induced such antigen expression in a small proportion of the uninfected cells. Thus, there was no evidence for global shut-off of astrocyte proteins by VZV infection, although this possible mechanism has not been excluded in the current study.

In order to determine the stage of the viral lytic cycle at which the apparent down-regulation of GFAP occurs, cultures were infected in the continuous presence of acyclovir (25 μg/ml). The late VZV genes coding for many structural proteins such as gpI are not synthesized until viral DNA replication starts. Since acyclovir inhibits viral DNA replication, late proteins should not be made in the presence of this drug. In acyclovir-treated cultures, no gpI staining was seen at 3 or 7 days postinfection, and only minimal cpe was noted compared with infected cultures not exposed to acyclovir. As expected, some staining for IE 62 protein was detected in a small percentage of infected acyclovir-treated cells. The percentage of $GFAP^+$ cells in acyclovir-treated cultures was about 50% of the total population — similar to that seen in infected but untreated cultures — and there was a very similar pattern of GFAP staining to that observed in the infected, untreated cultures that had not received acyclovir. Differential patterns of gpI staining could obviously not be assessed. There was no independent effect of acyclovir on GFAP expression or morphology as assessed in uninfected control cultures that had been treated with acyclovir during this time. Thus, VZV-induced down-regulation of GFAP appears to be mediated by early rather than late events during the viral replication cycle.

The down-regulation of GFAP expression during VZV infection is an intriguing event per se. Whether this primarily reflects diminished synthesis or increased virally induced degradation of the glial intermediate filaments is unclear, but it does seem to represent a form of biologic uncertainty phenomenon. The present findings argue that caution should be exercised in using GFAP as an astrocytic marker during *in vitro* VZV infection, since the infection itself appears to alter and diminish astrocytic GFAP expression. The number of virally infected astrocytes may therefore be significantly underestimated. The extent to which this phenomenon occurs with this and other cell type-specific markers using other viruses and in other tissue culture systems is unclear, but a review of prior work reveals some nonquantitated evidence that a similar process occurs during HSV infection of human astrocytes (*15*). Such caution should probably also be exercised in interpreting GFAP expression in tissue sections obtained from individuals with CNS VZV infections where down-regulation of GFAP expression may also occur. Further, while appreciating the intrinsic difficulties of extrapolating results obtained from *in vitro* experiments to the *in vivo* situation, in view of the diverse functions of astrocytes in the CNS, such an alteration of astrocytic GFAP expression may indicate a possible mechanism of virally induced damage seen in certain VZV infections of the human nervous system.

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