VIROLOGY 195, 219–228 (1993)

Differential Induction of Cytokines by Primary and Persistent Measles Virus Infections in Human Glial Cells

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Received December 8, 1992; accepted March 18, 1993

The effect of measles virus (MV) infection on mRNA expression and protein synthesis of cytokines in human malignant glioma cell lines (D-54 and U-251) was investigated. Primary MV infections led in both cell lines to the induction of interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon-β (IFN-β), and tumor necrosis factor-α (TNF-α). In contrast, persistently infected astrocytoma lines continually produced IL-6 (two out of 12 lines high levels) and IFN-β, whereas only 1 out of 12 lines synthesized TNF-α and none IL-1β. The pathways for induction of IL-1β and TNF-α expression were not suppressed by the persistent MV infection, since IL-1β and TNF-α could be induced by external stimuli like diacylglycerol analog plus calcium ionophore. Interestingly, persistently infected astrocytoma cells synthesized considerably higher levels of IL-1β and TNF-α than uninfected cells after additional external induction. These results suggest that in the central nervous system (CNS) of SSPE patients a percentage of persistently infected astrocytes may continually synthesize IL-6 and IFN-β, and in the presence of additional external stimuli, as possibly provided by activated lymphocytes, might overexpress the inflammatory cytokines IL-1β and TNF-α. This may be of pathogenetic significance in CNS diseases associated with persistent MV infections.

INTRODUCTION

Measles virus (MV) is the etiologic agent of acute measles and of the central nervous system (CNS) diseases acute measles encephalitis, measles inclusion body encephalitis and subacute sclerosing panencephalitis (SSPE). The latter disease begins with subtle mental and behavioral changes and progresses to complex functional defects, coma, and finally death. The disease develops on the basis of a persistent MV infection of neural cells and progresses in the presence of a substantial intrathecal humoral immune response. Although the molecular basis of MV persistence has been well characterized recently (Baczko *et al.*, 1986, 1992; Cattaneo *et al.*, 1986, 1987, 1988; Billeter and Cattaneo, 1991; Schmid *et al.*, 1992; Schneider-Schaulies *et al.*, 1989, 1990, 1992; ter Meulen *et al.*, 1983), little is known about the mechanisms which lead to cellular dysfunctions in the brain. In this context, cytokines expressed by infected brain cells may play a decisive role in the pathogenesis of SSPE not only during initial stages of the infection, but also influencing viral spread, viral gene expression, brain cell functions, and the intrathecal immune response (compare: Campbell, 1991).

In SSPE patients, where neurons and glial cells are the target cells for MV, elevated levels of IFN-α/β in the cerebrospinal fluid (CSF; Joncas *et al.*, 1976) and TNF-α positive cells with the morphology of astrocytes (Hofman *et al.*, 1991) have been detected. In addition, perivascular infiltrates of activated lymphocytes and macrophages are present in SSPE brain lesions synthesizing IFN-γ and TNF-α (Hofman *et al.*, 1991). However, data obtained from human brains reflect only final stages of SSPE and no information is available about the expression of cytokines and their cellular sources during early phases of the disease, when virus is spreading in the brain.

Tissue culture and animal model systems for cytokine expression in other CNS infections revealed a complex virus and target cell type-dependent pattern of cytokine induction by neural cells (Lieberman *et al.*, 1989; Shankar *et al.*, 1992). Many data about cytokine expression by human brain cells accumulated for the infection with human immunodeficiency virus-1, where, in contrast to SSPE, mainly CD4-positive microglial cells take up and propagate the virus (Jordan *et al.*, 1991; Johnson *et al.*, 1988). Infected microglial cells have been found to be the source of IL-1, IL-6, granulocyte-macrophage colony stimulating factor, and TNF-α (Merrill and Chen, 1991). Interesting differences in the cytokine expression following acute and persistent infections in the CNS have been found in relation to the genetic background of the host. In mice persistently infected with lymphocytic choriomeningitis virus (LCMV), significant levels of IL-6 could be detected in the CSF in high-responder (NMRI), but not in low-responder (CBA/J) strains (Moskophidis *et al.*,

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0042-6822/93 $5.00

1991). However, in acute LCMV infection, in both strains high levels of IL-6 in CSF and serum were found (Moskophidis *et al.*, 1991). The cellular source of IL-6 detected in mouse brains was identified as LCMV-infected astrocytes and microglial cells (Frei *et al.*, 1989). This interesting difference between acute and persistent infections suggest a linkage between cytokines and the pathogenesis of viral CNS infections.

In MV infection it is unknown which sets of cytokines are induced by primary or persistent infections of human glial cells and how expression of these cytokines might influence the immune response and cellular functions in the brain. In order to investigate the effects of MV on human brain cells, we studied the human malignant glioma cell lines D-54 and U-251, which show glial morphology and express markers of primary glial cells (Bigner *et al.*, 1981; Wikstrand *et al.*, 1985). We compared the cytokine expression after primary MV infection and in persistently infected clones of astrocytoma cells and found that persistently infected glioma cells synthesize less cytokines than after primary infection. However, in the presence of external stimulation, such as TPA and calcium ionophore, considerably higher levels of IL-1β and TNF-α were found in persistently infected cells than in uninfected cells. This phenomenon may be of pathogenetic significance of measles virus infection in the CNS.

MATERIALS AND METHODS

Propagation of measles virus

MV was prepared using Vero cells infected with a multiplicity of infection (m.o.i.) of 0.01 for 4 days at 33° followed by a one-time freezing/thawing of the cell monolayer in DMEM containing 5% FCS. For measurement of virus titers of persistently infected clones, 10^6 cells were cultured with 5 ml medium for 3 days and supernatants were removed for determination of cell-free virus. Cell-bound virus was prepared by freezing and thawing cell monolayers once with an equal volume of fresh medium that was removed for measuring free virus. Cell-bound and cell-free MV was titrated by end-point titration in triplicate on Vero cells (48-well plates) and averaged.

Culture and infection of glioma cells

The human malignant glioma cell lines D-54 and U-251 (Bigner *et al.*, 1981) were cultured in Dulbecco's minimal Essential medium (DMEM) containing streptomycin (100 μg/ml), penicillin (100 U/ml), and 5% fetal calf serum (FCS) at 37° and 10% CO_2. For the induction of cytokines after primary infections, cells were infected with a m.o.i. of 5 with MV (measles virus strain Edmonston). MV was adsorbed to monolayers of cells for 2 hr at 37° in DMEM containing 0.2% FCS. After adsorption, monolayers were washed with phosphate-buffered salt solution (PBS) at 37° and cells were cultured in DMEM containing 5% FCS.

Preparation of persistently infected clones and induction of cytokines

U-251 cells were subcloned by limiting dilution on microtiter plates and individual clones used to establish cell lines persistently infected with MV. U-251 clones 1-25 were infected with a low multiplicity (m.o.i. = 0.001). After lysis of most cells and further culture, clones of single surviving cells were allowed to grow out and were analyzed for viral persistence. Persistently infected cells were cultured in DMEM containing 10% FCS.

To test the inducibility of cytokines in persistently infected clones, the following substances were added to the culture medium: the diacylglycerol analog 12-*O*-tetradecanoyl phorbol myristat acetat (TPA, 15 n*M*; Sigma) plus the calcium ionophore A23187 (0.5 μ*M*; Sigma), indomethacin (10 μg/ml; Sigma), cycloheximide (5 μg/ml; Sigma), and bacterial lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (10 μg/ml; Sigma).

Immunofluorescent staining and flow cytometry

Flow cytometric characterization of infected astrocytoma cells was performed with mouse monoclonal antibody L77 against MV hemagglutinin (H) protein, or unrelated mouse IgG as negative control and fluorescein-conjugated rabbit anti-mouse Ig (Dako) as the second antibody. Cells (2×10^5/tube) were harvested and washed with FACS buffer (calcium and magnesium free PBS containing 0.2% BSA and 0.01 *M* NaN_3). After incubation with the first antibody diluted in FACS buffer on ice for 45 min, cells were washed three times with FACS buffer, incubated for 45 min with the second antibody, washed three times, and analyzed with a FACScan flow cytometer (Becton Dickinson).

ELISA

Enzyme-linked immunosorbant assays (ELISAs) for human IL-1β (IL-1β ELISA Kit, Cistron Biotechnology and Biochrom), IL-6 (IL-6 ELISA Kit, Dianova and Biochrom) and TNF-α (TNF-α ELISA Kit, Biomar and Biochrom) were performed as described in the manufacturers manuals. The evaluation of microtiter plates was performed automatically with an ELISA-reader (Bio-Rad) determining the optical density at 492 nm and standardized to samples of cytokines delivered by the manufacturers.

RNA preparation and Northern blot

Glioma cells were lysed *in situ* by 4 *M* guanidinium rhodanid buffer and total cellular RNA was pelleted through a cesium chloride cushion as described (Chirgwin *et al.*, 1979; Baczko *et al.*, 1984). Poly(A)$^+$ RNA was selected using oligo-dT-cellulose (Sigma). RNA (5 μg poly(A)$^+$ RNA per lane) was separated on 1.5% agarose gels containing 6.3% formaldehyde, blotted on Hybond-N filters (Amersham), and cross-linked with uv light (0.6 Joule/cm^2). The hybridization probes were for IL-1α: the 1.8-kb *Hin*dIII fragment of human IL-1α cDNA; IL-1β: the 1.5-kb *Pst*I fragment of human IL-1β cDNA; IL-3: the 0.9-kb *Xho*I fragment of gibbon IL-3 cDNA; IL-6: the 1.1-kb *Eco*RI fragment of human IL-6 cDNA; TNF-β: the 1.3-kb *Xho*I fragment of human TNF-β cDNA (the corresponding plasmids were a gift from Dr. Capalucci, Genetics Institute, Cambridge, MA); IFN-α: the 1.0-kb *Pst*I fragment of human IFN-α cDNA; IFN-β: the 1.1-kb *Hin*dIII fragment of human IFN-β cDNA (a gift from Dr. Zavatsky, Deutsches Krebsforschungs Zentrum, Heidelberg, Germany); TNF-α: the 1.3-kb *Xho*I fragment of human TNF-α cDNA (a gift from Dr. Tavernier, Roche Research, Gent, Belgium); GAPDH: the 1.4-kb *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase cDNA. The hybridization probes were radioactively labeled with [^{32}P]dCTP using a random-primed labeling kit (Boehringer-Mannheim). Blots were exposed to a screen for qualitative and quantitative evaluation with a phosphor imager (Molecular Dynamics) and to X-ray films.

Detection of MxA by Western blot

Supernatants of infected Astrocytoma clones cultured for three days and controls were harvested and added to human embryonic lung (HEL) fibroblasts in the presence of neutralizing anti-MV-H monoclonal antibodies K83 (0.5 μg/ml) for 48 hr. Recombinant human IFN-α (recombinant human IFN-α2a, Roferon-A3, Hoffmann–La Roche) was used as positive control. HEL cells (5 × 10^5) were dissolved in 300 μl sample buffer (80 m*M* Tris, pH 6.8, 2% SDS, 5% glycerol, 3.3% β-mercaptoethanol, and 0.02% bromophenol blue). Extracts were separated on 10% polyacrylamide gels containing SDS and blotted on nitrocellulose filters (Schleicher and Schüll) with semi-dry blotting chambers as described (Kyhse-Andersen, 1984). Filters were blocked with 10% dry milk powder in TBS (10 m*M* Tris, pH 7.2, 0.9% NaCl, 0.5% Tween) over night, incubated with monoclonal antibodies to human MxA p78 (a gift from Dr. Horisberger, Ciba-Geigy, Basel) for 1 hr, washed, and incubated with sheep anti-mouse Ig-HRP (Amersham). After washing, filters were rinsed in a peroxidase-sensitive luminescent solution (ECL-system, Amersham) and exposed to X-ray films for 10 to 20 sec.

TABLE 1

PERCENTAGES OF MV-POSITIVE CELLS AND TITERS OF INFECTIOUS MV IN CULTURES OF PERSISTENTLY INFECTED U-251 CELL CLONES

Cell line	Percentage infected cells[a]	MV titer (PFU/ml)[b]	
		In supernatants	Cell-bound
U-251 uninfected	0.8[c]	ND	ND
U-251 + MV[d]	86.5	1 × 10^6	5 × 10^6
U-251-p1	97.8	5 × 10^2	1 × 10^3
U-251-p4	85.2	5 × 10^1	1 × 10^2
U-251-p5	87.8	1 × 10^3	1 × 10^4
U-251-p7	99.3	5 × 10^2	1 × 10^3
U-251-p8	97.9	1 × 10^2	1 × 10^3
U-251-p9	93.5	5 × 10^2	5 × 10^3
U-251-p10	95.3	5 × 10^2	1 × 10^3
U-251-p12	91.6	1 × 10^3	1 × 10^4
U-251-p16	99.5	5 × 10^2	5 × 10^3
U-251-p19	98.7	5 × 10^2	5 × 10^3
U-251-p22	98.0	5 × 10^2	1 × 10^3
U-251-p23	75.6	1 × 10^2	5 × 10^2

Note. nd, not done.

[a] The percentage of positively stained cells was determined by flow cytometry. Cells were stained with anti-MV-hemagglutinin (H) monoclonal antibody L77.

[b] The titers were determined by end-point titration on Vero cells.

[c] Uninfected U-251 cells were used as negative control in flow cytometry.

[d] U-251 cells primarily infected with MV (strain Edmonston; m.o.i. = 1.0) for 48 hr.

RESULTS

Measles virus infection of human glioma cells

Infection of U-251 and D-54 cells with MV (m.o.i. = 1) led in both cultures to giant cell formation, synthesis of high titer of progeny virus (approximately 10^6 PFU/ml) and lysis of cells after 2 to 5 days. Infection with a low multiplicity (m.o.i. = 0.001) also resulted in the development of a cytopathic effect, which killed most of the cells, but in the case of U-251 cells, clones of single surviving persistently infected cells grew out, which could never be observed with D-54 cells.

Since persistent MV infection could be established reproducibly in U-251 astrocytoma cells, we assessed the question whether only a small subpopulation of U-251 might be selected during the establishment of the persistent infection, or whether all U-251 cells have the intrinsic potential to become persistently infected. For this purpose, we subcloned U-251 and subsequently infected these clones with MV. From 25 infected subclones, we obtained 12 surviving cell lines persistently infected with MV, designated U-251-p1, -p4, -p5, -p7, -p8, -p9, -p10, -p12, -p16, -p19, -p22, and -p23. As shown in Table 1, a high percentage of the cells in these cultures expressed measles antigens on their surface (75.6 to 99.5%) when analyzed by flow cytometry after staining with monoclonal antibody to

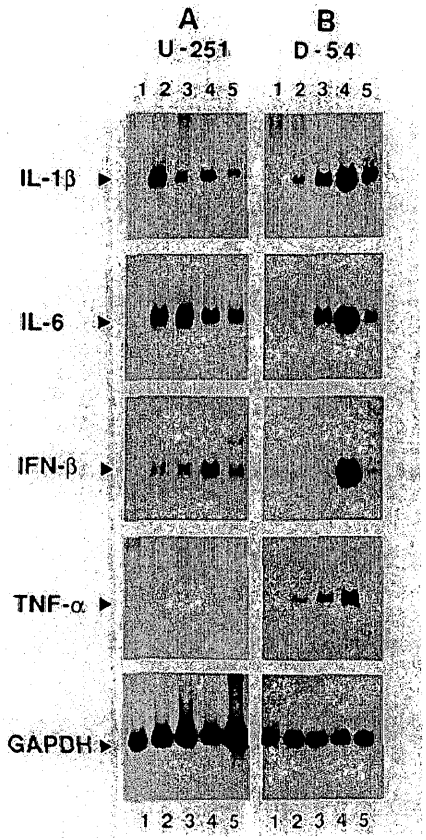

FIG. 1. Induction of cytokine-specific mRNAs in human glial cells after primary infection with MV. U-251 (A) and D-54 cells (B) were infected with MV (m.o.i. = 5) for 0, 6, 12, 24, and 48 hr (lanes 1 to 5, respectively). Poly(A$^+$)-RNA was prepared, blotted on Hybond-N filters, and hybridized to ^{32}P-labeled probes for IL-1β, IL-6, IFN-β, TNF-α, and GAPDH as control for the quality and the amount of blotted RNA.

MV hemagglutinin protein. Considerable amounts of infectious MV were produced by all persistently infected cell clones as free and cell-bound virus, although to lower titers when compared to primary infections (Table 1). All 12 persistently infected cell lines carried MV when cultured for at least 30 passages.

Cytokine induction after primary infection with measles virus

To investigate the effect of MV on the expression of cytokines in human glioma cells, cells were exposed to a high multiplicity of MV (m.o.i. = 5), in order to infect all cells simultaneously. Cellular RNA was extracted 6, 16, 24, and 48 hrs after infection as well as from uninfected cells. Northern blots were hybridized with probes for IL-1α, IL-1β, IL-3, IL-6, IFN-α, IFN-β, IFN-γ, TNF-α, and TNF-β. Cellular mRNAs of correct sizes were found to hybridize to probes for IL-1β, IL-6, IFN-β, and TNF-α (Fig. 1). The quantities and kinetics of the cytokine-specific mRNAs in U-251 cells (Fig. 1A) and D-54 cells (Fig. 1B) were characteristically different. In U-251 cells, cytokine induction was found to occur earlier than in D-54 cells. This correlated well with the observation that progression of MV infection is generally faster in U-251 cells than in D-54 cells. High levels of mRNAs of IL-1β, IL-6, and IFN-β were expressed in U-251 cells after 6 hr of infection, whereas in D-54 cells these cytokines were expressed maximally after 24 hr. D-54 cells showed strong signals for TNF-α mRNA after 12 and 24 hr following infection, which were only weakly detectable in U-251 cells. After 48 hr postinfection the levels of cytokine mRNAs decreased coinciding with the onset of giant cell formation and subsequent lysis of the glioma cells.

The presence of IL-1β, IL-6, and TNF-α as proteins in supernatants of cell cultures was tested with enzyme-linked immunosorbant assays. The concentrations of these cytokines were determined by comparison to standard signals and are given in Table 2. After 48 and 72 hr of infection, high levels of IL-6 had accumulated in supernatants of both glioma cell lines. Levels of IL-1β and TNF-α were relatively low (Table 2). These data correlated well with the levels of mRNAs obtained by Northern blots.

The human interferon-inducible MxA protein is exclusively induced by type 1 interferons, and therefore can be used as indicator for the presence of IFN-α/β in cell supernatants (Horisberger and Gunst, 1991; Simon *et al.*, 1991; von Wussow *et al.*, 1990). In order to find out whether the MV-infected cultures synthesize biologically active IFN-α/β, we determined the induction of MxA protein in HEL cells treated with supernatants of infected cells for 48 hr by Western blot. Neutralizing amounts of the anti-MV monoclonal antibody K83 were added to the supernatants and controls 30 min before

TABLE 2

CYTOKINE CONCENTRATIONS IN SUPERNATANTS OF PRIMARILY INFECTED GLIOMA CELLS

Cell line	Time p.i.[a] (hr)	Concentration (pg/ml) of [b] IL-1β	IL-6	TNF-α
U-251	0	–[c]	–	–
	24	–	722 ± 116	–
	48	35 ± 10	3628 ± 343	–
	72	50 ± 21	3138 ± 233	30 ± 18
D-54	0	–	–	–
	24	–	314 ± 34	–
	48	–	1310 ± 43	–
	72	55 ± 18	2622 ± 177	64 ± 15

[a] Cells (10^6/5 ml medium) were infected with MV with a m.o.i. of 1.0 for indicated times.

[b] Quantified by ELISA compared to standard proteins as provided by the manufacturers.

[c] Below or equal to background values detected in medium (≤30 pg/ml; $n = 3$).

addition of the supernatants to the HEL cells to exclude induction of MxA by infection with MV. The MxA protein could be detected as a 78-kDa band induced by the supernatant of 48 hr MV infection (m.o.i. = 1) of U-251 cells (Fig. 2A, lane 2). Following treatment of U-251 cells as control with 0.1 to 1000 U/ml recombinant IFN-α for 48 hr, MxA was detected already with 0.1 U/ml IFN-α (Fig. 2A, lanes 3–8). According to the titration curve, the supernatant of 48 hrs infected U-251 cells contained approximately 10 U/ml type I interferon. D-54 cell supernatants also induced high levels of MxA protein after 48 hr of treatment of HEL cells (not shown) indicating that biologically active IFN-α/β was synthesized in both human glioma cells in the course of MV infection.

Cytokine expression in persistently MV-infected cells

In order to analyze the cytokine expression in persistently infected U-251 cells at the RNA level, poly(A)$^+$ RNA from the 12 persistently infected U-251 cell clones was prepared. Northern blots revealed that these cells generally expressed lower levels of cytokine-specific mRNAs than primarily infected U-251 cells with considerable variations among the individual clones, although all clones were kept in culture under the same conditions. Expression of IL-1β mRNA was found at a very low level or below the detection limits of the Northern blot (Fig. 3). Relatively strong signals were found for IL-6 mRNA in clones U-251-p9 and -p16 (Fig. 3, lanes 6 and 9). The remaining clones showed weak but detectable signals for IL-6 mRNA. For IFN-β mRNA, weak to hardly-detectable signals were found. Clear signals for TNF-α mRNA were only seen in clones U-251-p1 and -p8 (Fig. 3, lanes 1 and 5).

The concentrations of IL-1β, IL-6, and TNF-α as proteins in supernatants of the persistently infected clones were determined by ELISAs (Table 3). The levels of IL-1β in the supernatants were below the detec-

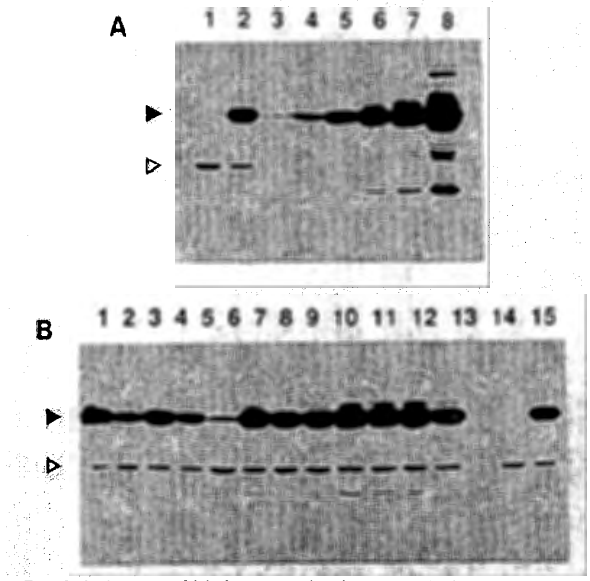

FIG. 2. Induction of MxA expression in HEL cells by supernatants of infected U-251 cells and IFN-α. (A) Western blot analysis of human MxA in HEL cells treated with supernatants of uninfected U-251 cells (lane 1) and of 48-hr MV-infected U-251 cells (lane 2), and HEL cells stimulated with human recombinant IFN-α 0.1, 0.5, 1, 10, 100, 1000 U/ml (lanes 3–8, respectively). The MxA protein (closed triangle; 78 kDa) was detected with monoclonal antibody P78, HRP-conjugated second antibodies, and a luminescence reaction (see Materials and Methods). Lower molecular weight bands in lanes 1 and 2 are anti-MV-H antibodies, which were added for neutralization of MV (open triangle; 50 kDa) and are detected by the second antibody. Additional bands in lanes 6–8 are degradation products and complexes of MxA. (B) Western blot analysis of human MxA (closed triangle) induced by supernatants of persistently infected cell clones U-251-p1, -p4, -p5, -p7, -p8, -p9, -p10, -p12, -p16, -p19, -p22, and -p23 (lanes 1–12, respectively); uninfected U-251 cells (lanes 13 and 14); and 48-hr MV-infected U-251 cells (lane 15, same supernatant as in (A) lane 2). For neutralization of infectious MV, anti-MV-H antibodies were added to the supernatants in lanes 1–12, 14, and 15 and are detected by the second antibody (open triangle).

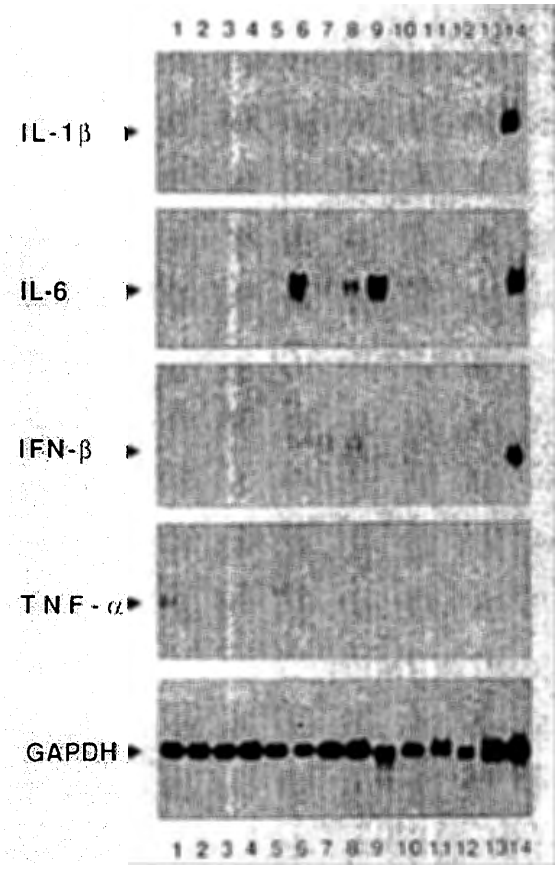

FIG. 3. Expression of cytokine-specific mRNAs in persistently infected U-251 cell clones. Poly(A$^+$)-RNAs of the persistently infected clones U-251-p1, -p4, -p5, -p7, -p8, -p9, -p10, -p12, -p16, -p19, -p22, and -p23 (lanes 1–12, respectively); uninfected U-251 cells (lane 13); and 6-hr (IL-1β, IL-6, and TNF-α) or 48-hr (IFN-β) MV-infected U-251 cells (lane 14) were isolated, blotted, and hybridized to probes for IL-1β, IL-6, IFN-β, TNF-α, and GAPDH as control of the quality and amount of blotted mRNA.

TABLE 3

CYTOKINE CONCENTRATIONS IN SUPERNATANTS OF PERSISTENTLY INFECTED ASTROCYTOMA CELLS

Cell line	Concentration (pg/ml) of [a]		
	IL-1β	IL-6	TNF-α
U-251 uninfected	–[b]	–	–
U-251-p1	–	120 ± 58	760 ± 285
U-251-p4	–	45 ± 30	–
U-251-p5	–	105 ± 75	–
U-251-p7	–	100 ± 50	–
U-251-p8	–	190 ± 15	–
U-251-p9	–	3260 ± 195	–
U-251-p10	–	100 ± 50	–
U-251-p12	–	262 ± 21	–
U-251-p16	–	4086 ± 254	–
U-251-p19	–	303 ± 22	–
U-251-p22	–	715 ± 62	–
U-251-p23	–	50 ± 32	–

[a] Supernatants were taken after 3 days of culture of 10^6 cells/5 ml medium. Standard proteins of the manufacturers were used for quantification.

[b] Below or equal to background values detected in medium (≤30 pg/ml; $n = 3$).

tion limit, whereas IL-6 was secreted in measurable amounts. The levels of IL-6 proteins in the supernatants of the different cell clones correlated with levels of IL-6 mRNAs detected by Northern blots. Clones U-251-p9 and -p16 constantly produced as high levels of IL-6 as did U-251 cells after primary infection with MV (Table 3). Clone U-251-p1, which also expressed a substantial level of TNF-α mRNA, released a considerable amount of TNF-α protein into the supernatants (760 ± 285 pg/ml; Table 3). All other persistently infected clones did not synthesize measurable amounts of TNF-α protein.

The presence of biologically active IFN-α/β in the supernatants was again analyzed by Western blot with extracts from 48-hr-treated HEL cells (Fig. 2). All supernatants of persistently infected clones induced MxA. The levels of induced MxA correlated only partially to the levels of IFN-β specific mRNAs (Fig. 3), where mRNA levels in some cell clones were below the detection limit of the Northern blots. These data indicate that persistently infected astrocytoma cells synthesize continually the cytokines IL-6 and IFN-β, although to varying amounts, but failed to express IL-1β, and, with one exception, also TNF-α.

Reactivity of persistently infected astrocytoma cells to additional external stimuli

The observed absence of IL-1β and TNF-α in supernatants of cells persistently infected with MV could be a consequence of the persistent infection inhibiting signal transduction pathways involved in the induction of the corresponding genes. Alternatively, the downregulation may be due to a lack of inducing signals for transcription and expression of these genes. These hypotheses could be tested using additional external stimuli. For this purpose, U-251 and the persistently infected clones U-251-p8, -p9, and -p10 were treated with substances known to stimulate the transcription or mRNA stability of IL-1β and TNF-α, namely the tumor promoter TPA, calcium ionophore A23187, LPS, and cycloheximide. Northern blots revealed that in uninfected (Fig. 4A) as well as in persistently infected cells (Fig. 4B; clone U-251-p8) high levels of IL-1β and IL-6 mRNAs could be stimulated by TPA-plus calcium ionophore (Figs. 4A and 4B, lane 1). In contrast, only faint amounts of TNF-α mRNA were induced in uninfected U-251 cells, whereas the persistently infected clones expressed high levels (Figs. 4A and 4B, lane 1). Pretreatment of cells with indomethacin, a blocker of the cyclooxygenase-derived eicosanoid synthesis in astrocytes (Fontana *et al.*, 1982; Hartung *et al.*, 1989),

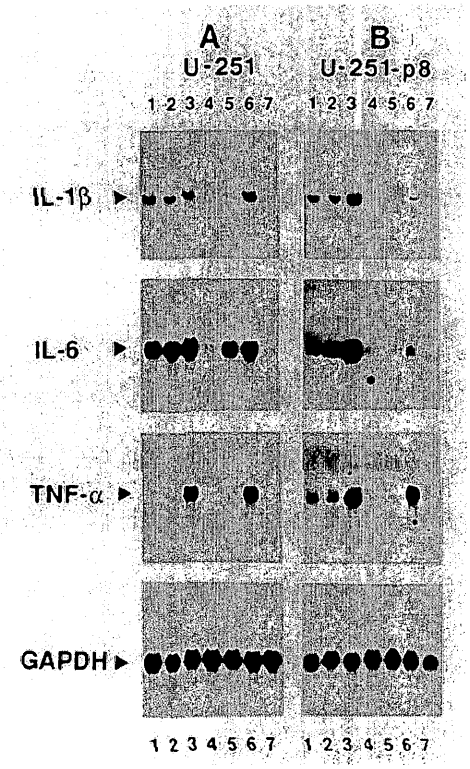

FIG. 4. Expression of cytokine-specific mRNAs in externally stimulated uninfected U-251 and persistently infected U-251-p8 astrocytoma cells. U-251 (A) and U-251-p8 (B) cells were incubated in the presence of inductors, and poly(A^+)-mRNAs were prepared and blotted on Hybond-N filters. The lanes contain RNA from cells treated with 6 hr TPA plus calcium ionophore (lane 1); 24 hr indomethacin and 6 hr TPA plus calcium ionophore (lane 2); 6 hr TPA plus calcium ionophore plus cycloheximide (lane 3); 6 hr LPS (lane 4); 24 hr indomethacin and 6 hr LPS (lane 5); 6 hr cycloheximide (lane 6); and medium (lane 7). Similar results were obtained with clones U-251-9 and -p10.

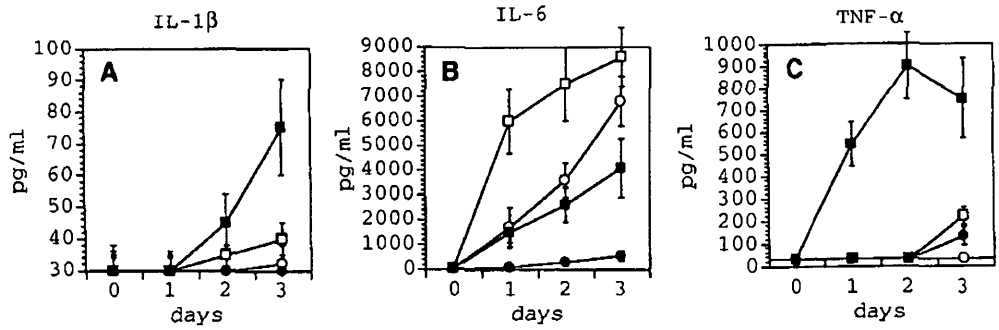

FIG. 5. Cytokine concentrations in stimulated uninfected astrocytoma cells and persistently infected clone U-251-p8. Concentrations of IL-1β (A), IL-6 (B), and TNF-α (C) in supernatants of uninfected U-251 cells (open symbols) and persistently infected U-251-p8 cells (closed symbols) were determined by ELISAs in three independent experiments. Cells were treated for 0, 1, 2, and 3 days with TPA plus calcium ionophore (squares), and LPS (circles). The scale starts with background values at 30 pg/ml.

did not alter the induction of mRNAs by TPA-plus calcium ionophore (Figs. 4A and 4B, lane 2). Cycloheximide enhanced the levels TNF-α mRNA in uninfected and persistently infected cells (Figs. 4A and 4B, lane 3), most likely due to its influence on the half-life of TNF-α mRNA (Bethea *et al.*, 1992; Lieberman *et al.*, 1990). LPS alone induced only low levels of mRNAs (Figs. 4A and 4B, lane 4). Pretreatment with indomethacin and subsequent stimulation with LPS led to high levels of IL-6 in uninfected U-251 cells, but not in persistently infected clones (Figs. 4A and 4B, lane 5). Cycloheximide alone for 6 hr induced IL-1β, IL-6, and TNF-α mRNA in U-251 cells and in persistently infected clones (Figs. 4A and 4B, lane 6). The same results as with clone U-251-p8 were found with clones U-251-p9 and -p10 (not shown). The induction of IL-6 mRNA in persistently infected cells was not as strong as in uninfected U-251 cells and dependent on the level of continued IL-6 expression in individual clones as shown in Fig. 3 and Table 3.

The levels of cytokines in supernatants of astrocytoma cells externally stimulated were determined by ELISA. The results for U-251 and U-251-p8 cells after 0, 1, 2, and 3 days of treatment with TPA plus calcium ionophore, and LPS are shown (Fig. 5). Under these conditions, persistently infected cells synthesized much higher levels of IL-1β and TNF-α protein than uninfected control cells. In contrast, lower amounts of IL-6 were expressed by stimulated persistently infected clone U-251-p8 than in uninfected U-251 cells (Fig. 5). These data indicate that the down-regulation of IL-1β and TNF-α in persistently infected cells can be overcome by external stimuli, and that considerably higher levels of IL-1β and TNF-α protein are synthesized compared to stimulated uninfected astrocytoma cells.

DISCUSSION

In this study, the effect of primary versus persistent MV infections on the cytokine expression in human glioma cells has been investigated in tissue culture. Primary MV infection of human glioma cells transiently induced the expression of the cytokines IL-1β, IL-6, IFN-β, and TNF-α (Table 4). With the exception of IL-1, similar results have been obtained for primary rat astrocytes infected with Newcastle disease virus (Lieberman *et al.*, 1989), or in fibroblasts after infection with MV and vesicular stomatitis virus (VSV; van Damme *et al.*, 1989). Moreover, IL-1, TNF-α, IFN-α/β, and IL-6 have also been found in CSF and serum samples during acute and persistent infections with HIV in man and VSV or LCMV in mice (Frei *et al.*, 1989; Merrill and Chen, 1991; Moskophidis *et al.*, 1991). Thus, synthesis of these cytokines seems to be a common feature of viral diseases of the CNS.

TABLE 4

SUMMARY OF CYTOKINE EXPRESSION IN MEASLES VIRUS INFECTED HUMAN GLIOMA CELLS

Cytokine	No infection	Primary infection	Persistent infection	Pers. infect. plus stim.[a]
IL-1α	–	–[b]	–	nd
IL-1β	–	+	–	++
IL-3	–	–	–	nd
IL-6	–	+++	++[c]	++
IFN-α	–	–	–	nd
IFN-β	–	++	+	nd
TNF-α	–	+	–[d]	++
TNF-β	–	–	–	nd

Note. nd, not done.

[a] Persistently MV-infected clones stimulated with TPA plus calcium ionophore.

[b] Presence of cytokines was based on Northern blots or ELISAs as far as performed (IL-1β, IL-6, and TNF-α). Levels of cytokines are indicated by – (below detection limit), + (30–100 pg/ml), ++ (101–1000 pg/ml), and +++ (>1000 pg/ml).

[c] Levels of IL-6 are variable depending on the individual clone of persistently infected astrocytoma cells.

[d] Persistently infected U-251 clones did not secrete TNF-α with the exception of clone U-251-p1 (Table 3).

In contrast to the short lasting primary infection, we found that persistently infected clones of astrocytoma cells continually expressed various amounts of IL-6 and IFN-β, but not IL-1β, and TNF-α (with exception of one clone; Table 3). In brain tissue, elevated levels of IL-6 during persistent infections may influence the intrathecal differentiation of B lymphocytes and enhance the occurrence of oligoclonal antibodies as found in the CSF of SSPE patients (ter Meulen *et al.*, 1983). As a consequence of high levels of MV antibodies, a down-regulation of MV replication occurs by the mechanism of antibody-mediated modulation as shown in *in vitro* and *in vivo* studies (Barret *et al.*, 1985; Fujinami and Oldstone, 1980; Schneider-Schaulies *et al.*, 1992; Liebert *et al.*, 1990). Presence of IFN-α/β in serum and CSF of SSPE patients has been reported (Joncas *et al.*, 1976) and has also been suggested to lead to the restriction of MV gene expression in SSPE brains (Carrigan and Knox, 1990; Fujii *et al.*, 1988). Therefore, IFN-α/β and IL-6 may play an important role in restricting replication and spread of MV in the human brain.

With exception of clone U-251-p1, which continually produced approximately 760 pg/ml TNF-α, all other persistently infected cells did not synthesize detectable amounts of the cytokines IL-1β and TNF-α. Usually, transcription of the TNF-α gene in astrocytes is a transient event even in the presence of stimulating molecules and its mRNA decreases with a short half-life (Bethea *et al.*, 1992). At present, we can not explain the presence of TNF-α in just one persistently infected clone. Since in most of our persistently infected clones TNF-α mRNA and protein was not detectable, we believe that the persistent infection itself is not sufficient for continuous TNF-α induction. However, these cells remain in a preactivated state highly responsive to additional external stimuli leading to overexpression of IL-1β and especially TNF-α. It is possible that persistent MV infection leads to a constantly increased transcription rate of IL-1β and TNF-α, while mRNA molecules cannot be detected due to their short half-lives. Interestingly, it was found in human monocytes that MV infection causes increased IL-1β and TNF-α transcription and reduces the half-life of TNF-α, but not IL-1β mRNA (Leopardi *et al.*, 1992). Since very low levels of IL-1β can stimulate TNF-α transcription in astroglioma cells (Bethea *et al.*, 1992), we can not exclude that constant expression of IL-1β in persistently infected cells below the detection limit of ELISAs is responsible for ongoing TNF-α transcription. Signals elongating the very short half-life of TNF-α mRNA, possibly mediated via protein kinases as shown for primary rat astrocytic cultures (Lieberman *et al.*, 1990), could then lead to high levels of mRNA within a short time. *In vivo*, such signals might be provided in the brain by infiltrating cells of the immune system. It is conceivable, that during initial stages of a persistent infection IL-1 and TNF-α expression is low, whereas after the induction of a virus specific immune response and release of interleukins, high levels of both cytokines might be induced in persistently infected astrocytes.

Overexpression of IL-1β, and even more TNF-α, by persistently MV-infected astrocytoma cells in the presence of additional stimuli is an interesting finding, since both cytokines have pleiotropic effects in the CNS. Among the effects of IL-1 are the regulation of the body temperature; food uptake and sleep (Plata-Salaman, 1991); induction of other cytokines like IL-6, TNF-α, and nerve growth factor (Bethea *et al.*, 1992; Lindholm *et al.*, 1987; Yasukawa *et al.*, 1987); the stimulation of surface molecules together with other cytokines (Frohman *et al.*, 1989; Massa *et al.*, 1987); chronic inflammatory effects (Martiney *et al.*, 1992); and effects together with TNF-α on the proliferation and differentiation of glial cells (Barna *et al.*, 1990; Giulian and Lachman, 1985; Merrill, 1991). TNF-α alone can stimulate astrocytes to proliferation (Merrill, 1991), which may lead to astrogliosis as observed in SSPE brains. Our data suggest that both factors, the persistent infection of glial cells and additional stimuli possibly provided by the immune system, could synergistically lead to the release of high levels of the inflammatory cytokines IL-1 and TNF-α in the brain of SSPE patients.

ACKNOWLEDGMENTS

The authors thank M. Bayer, S. Löffler, and K. Pech for excellent technical assistence: Dr. Bigner (Durham, USA) for providing us with the glioma cell lines; Dr. Capalucci (Genetics Institute, Cambridge, USA), Dr. Zavatsky (Deutsches Krebsforschungs Zentrum, Heidelberg, Germany), and Dr. Tavernier (Roche Research, Gent, Belgium) for the cytokine probes; and Dr. Horisberger (Ciba-Geigy, Basel, Switzerland) for the MxA specific antibody. This work was supported by the Deutsche Forschungsgemeinschaft and Bundesministerium für Forschung und Technologie.

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