

Tumour necrosis factor- α , interferon- γ and interferon- β exert antiviral activity in nervous tissue cells

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The individual and synergistic antiviral effects of cytokines released by infiltrating immune cells or by cells of the nervous system may play an important role in inhibiting virus spread during infections of the central nervous system (CNS). We examined the antiviral activity against the neurotropic pseudorabies virus (PRV) of interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α), and combinations of these cytokines, as compared to that of IFN- β , in rat nervous tissue cells. PRV replicated efficiently in all neural cell types tested, including neurons, astrocytes and oligodendrocytes. The inhibitory effects were determined by quantifying the inhibition of virus plaque formation, yields of infectious virus at various times after infection

and synthesis of viral proteins. At a low m.o.i., IFN- γ and IFN- β inhibited viral plaque formation in all cell types; TNF- α was effective only in astrocytes but showed synergy with IFN- γ . At a higher m.o.i., IFN- β inhibited yields of infectious virus more effectively than IFN- γ , whereas TNF- α had no effect on virus yields and was only marginally synergistic with the antiviral activity of IFN- γ . The yield-reduction assays correlated well with cytokine-induced inhibition of viral protein synthesis. Our results show that both IFN- γ and IFN- β can induce a state of antiviral resistance in neural cells whereas TNF- α is effective only in astrocytes at low m.o.i.; they suggest an antiviral role of cytokines in the immune response to virus infections of the CNS.

Introduction

Cytokines are involved in the initiation, modulation and prolongation of antiviral immune responses. They induce or increase the expression of major histocompatibility complex (MHC) and adhesion molecules and regulate the development and activity of immune effector cells. Furthermore, many cytokines may induce the production of other cytokines and affect their activity either synergistically or antagonistically. Within this complex network, tumour necrosis factor (TNF), interferon- γ (IFN- γ) and IFN- α/β form a subset of molecules which, in addition to their capacity to accelerate immune functions, are also known to act as antiviral effector molecules, either via induction of an antiviral state in the target cell (Lewis, 1982) or via specific lysis of infected cells (Aderka *et al.*, 1985; Mestan *et al.*, 1986; Wong & Goeddel, 1986). These characteristics may play an important role in inhibiting virus spread *in vivo*.

In vivo studies have provided evidence that IFN- γ produced locally in infected tissues by herpes simplex virus (HSV)- and vaccinia virus-specific cytotoxic T cells is of importance for protection (Sethi *et al.*, 1983; Bennink

et al., 1985). The *in vitro* release of IFN- γ by protective T cells upon antigen recognition has been demonstrated for a variety of viruses (Morris *et al.*, 1982; Epstein *et al.*, 1972; Starr *et al.*, 1980; Valle *et al.*, 1975). A recent study using the lymphocytic choriomeningitis virus model demonstrated that the administration of antibodies to IFN- γ neutralized the *in vivo* antiviral activity of this molecule and facilitated virus replication (Leist *et al.*, 1989). Secretion of TNF and lymphotoxin by natural killer (NK) cells has been shown to mediate selective and direct cytotoxicity against virus-infected cells (Paya *et al.*, 1989). All these studies suggest that the limitation of viral replication *in vivo* exerted by T cells, NK cells or other immune cells depends on the release of cytokines, in particular IFN- γ and TNF. The pronounced antiviral synergism between IFN- γ and TNF (Wong *et al.*, 1988; Feduchi *et al.*, 1989) suggests that the presence of both factors, when released locally in the infected tissue, may contribute to the prevention of virus spread.

The antiviral role of cytokines present in the central nervous system (CNS) during virus-induced meningitis or encephalitis is unclear. The production of IFNs and TNF in the CNS can no longer be attributed exclusively

to infiltrating macrophages and lymphoid cells but must be due also to neural cells. Virus- or lipopolysaccharide-induced production of TNF and IFN- α/β has been observed recently in astrocyte cultures (Lieberman *et al.*, 1989; Tedeschi *et al.*, 1986) and stimulated microglial cells (Righi *et al.*, 1989; Frei *et al.*, 1987). Furthermore, TNF immunopositive astrocytes have been demonstrated histologically in brain lesions of multiple sclerosis patients (Hofman *et al.*, 1989). The lymphokine IFN- γ , which was thought to be exclusively produced by activated T lymphocytes and NK cells (O'Malley *et al.*, 1982; Handa *et al.*, 1983), has recently been demonstrated immunohistologically in neurons (Ljungdahl *et al.*, 1989) as well as in astrocytes adjacent to brain lesions in multiple sclerosis patients (Traugott & Lebon, 1988).

The release of antivirally active cytokines by infiltrating immune cells or by local neural cells may play a crucial role in inhibiting virus spread in the CNS. Recently we found that recombinant IFN- γ injected into the cerebroventricular system could protect rats against a lethal pseudorabies virus (PRV) infection (Schijns *et al.*, 1990). PRV, an HSV-related alphaherpesvirus, causes neurological disease in a variety of species and has been shown histologically to infect neurons as well as glial cells (Schijns *et al.*, 1989). In the present study we examined the synergistic and individual antiviral activity of IFN- γ and TNF- α , as compared to IFN- β , in rat spinal cord neurons and in astrocyte- or oligodendrocyte-enriched glial cells.

Methods

Virus. A field isolate of PRV was obtained from the Central Veterinary Institute, Lelystad, The Netherlands. The batches used in this study were passages 3 and 4 in rat embryo fibroblasts.

Cells. Rat embryo fibroblast (Ratoc) cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin [100 units (U)/ml] and streptomycin (100 μ g/ml).

Primary cultures of dissociated neurons were prepared from 15-day-old foetal Wistar rats (obtained from the Central Institute for Laboratory Animals, Zeist, The Netherlands) as previously described (Van der Neut *et al.*, 1990). Briefly, spinal cords were dissected and trypsinized after removing the meninges and dorsal root ganglia (15 min at 37 °C). Single cells were obtained by mechanical dissociation and cultured on poly-L-lysine-coated glass cover slips, in 35 mm plastic dishes or in 16 mm 24-well plates (Costar). Cells were maintained in a chemically defined medium (CDM) (Bottenstein & Sato, 1979).

Glial cell cultures were prepared as previously described (Koper *et al.*, 1984). Briefly, cerebra of 7-day-old Wistar rats were minced and incubated with trypsin (45 min at 37 °C). The tissue was then triturated in the presence of soybean trypsin inhibitor and sieved through a nylon screen to remove debris. The cell suspension was plated on poly-L-lysine-coated glass or plastic surfaces and cultured for 24 h in 10% FCS. One day after plating, the medium was replaced by CDM (Koper *et al.*, 1986). The development of glial cell cultures varies with the composition of the culture medium; addition of cytosine β -D-

arabinofuranoside (10^{-5} M) to CDM inhibits astroglial overgrowth resulting in cultures enriched for oligodendrocytes. Glial cells maintained in DMEM/10% newborn calf serum developed into astrocyte-enriched cultures (Koper *et al.*, 1986).

Cytokines. Recombinant rat IFN- γ and IFN- β produced by Chinese hamster ovary cells were purified as previously described (Van der Meide *et al.*, 1986; Dijkema *et al.*, 1985). Preparations were assayed in a c.p.e. inhibition assay on Ratec cells challenged with vesicular stomatitis virus. Activity is expressed in laboratory units standardized against a rat IFN reference (Schellekens *et al.*, 1980). The IFN preparations used had a specific activity of 4.0×10^6 U/mg protein. Recombinant murine TNF- α , kindly provided by Dr W. Fiers (Gent, Belgium), had a specific activity of 75×10^6 U/mg as assayed on L929 cells.

Conditions of cytokine treatment and infection. After 5 days in culture, dissociated spinal neurons, astrocytes and oligodendrocyte glial cells were treated with different doses of IFN, TNF- α , or combinations of TNF- α and IFN- γ for 24 h. The cultures were washed and infected with approximately 500 p.f.u./PRV well. The cells were washed 1 h after inoculation and fresh medium was added. To determine the amount of inhibition of plaque formation, cultures were fixed 24 h post-infection with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and subsequently permeabilized with 95% ethanol for 5 min. The antiviral effect was determined by quantifying antigen-containing plaques visualized by an immunoperoxidase technique (Schijns *et al.*, 1989). This was done to ensure that holes in the cell layer that were not due to viral attack were excluded from quantification and also to identify plaques which lacked morphological changes. At the chosen m.o.i. there was no risk of plaque overlap. Experiments were performed at least twice.

To determine the effects of cytokines on yields of infectious virus at various times after infection, the different neural cells were exposed to 10^3 U/ml IFN- γ or IFN- β , 10^2 U/ml TNF- α , or a combination of IFN- γ and TNF- α , for 24 h. After washing, cultures were infected with PRV at an m.o.i. of 1.0. At various times after infection, cell-bound virus and virus in the supernatant was collected and exposed to three cycles of freezing and thawing in medium containing 20% FCS. The concentration of infectious virus was titrated in Ratec cells. The experiments were performed at least twice.

Immunocytochemistry. Presence of PRV antigen was determined by an immunofluorescence or immunoperoxidase technique as described previously (Schijns *et al.*, 1989). Rabbit anti-glial fibrillary acidic protein (GFAP) serum (Dakopatts), a monoclonal anti-galactocerebroside (GalC) antibody (a gift from Dr M. Noble, London, U.K.), as well as mouse anti-neurofilament (NF) serum (NF 2F11; Monosan Sanbio) were used as cell-specific markers for astrocytes (Bigami *et al.*, 1972), oligodendrocytes (Raff *et al.*, 1983) and neurons (Van der Neut *et al.*, 1988), respectively. Double immunolabelling experiments were performed as previously described to identify the types of cells containing viral antigen (Noble & Murray, 1984). Preparations were examined and photographed using an epifluorescence microscope (Zeiss) with selective rhodamine and fluorescein filters.

Analysis of viral proteins in cytokine-treated cells. The different neural cells and the fibroblast cultures were exposed to 10^3 U/ml IFN- γ or IFN- β , 10^2 U/ml TNF- α , or combinations of IFN- γ and TNF- α , for 24 h. The cells were washed and infected with PRV at an m.o.i. of 1.0. At various times after infection, but 1 h before labelling, the medium was replaced by methionine-deficient DMEM followed by pulse-labelling with 60 μ Ci/ml [35 S]methionine. After 1 h the medium was removed and the cells were washed with PBS. Cells were lysed in 1 ml TES buffer (20 mM-Tris-HCl pH 7.5, 1 mM-EDTA, 100 mM-NaCl) containing 0.1% Triton X-100 and 2 mM-PMSF. Labelled proteins were analysed by electrophoresis in 15% SDS-polyacrylamide gels.

Results

Characterization of primary cultures of neural cells

At the time of infection, 5-day-old glial oligodendrocyte-enriched cultures consisted of between 70 and 90% GalC-positive (+) oligodendrocytes, between 10 and 30% GFAP-positive astrocytes and less than 1% other cells (predominantly microglial cells; identified morphologically), as determined by double immunofluorescence. Type 1 astrocyte-enriched glial cultures, maintained in 10% newborn calf serum, contained 98% astrocytes and 2% other cells (predominantly oligodendrocytes) 5 days after plating. Cultures of dissociated spinal cord cells consisted of 99% neurons, as determined by NF immunofluorescence staining. Only a few GFAP⁺ astrocytes (1%) and fibroblasts (<1%) were present.

Susceptibility of different neural cells to PRV infection

Since the various cell types of the CNS may differ in their susceptibility to PRV infection, we used double-labelling indirect immunofluorescence to identify infected cells in the heterogeneous primary cultures. Fig. 1 shows that NF⁺ neurons, GFAP⁺ astrocytes as well as GALC⁺ oligodendrocytes contain viral antigen. In astrocytes and fibroblasts a c.p.e. was observed; in the plaque centres syncytium formation was apparent whereas rounded cells were present in the periphery (not shown). In infected neuronal cells and oligodendrocytes no morphological changes were observed 24 h after infection at low m.o.i. At that time, however, PRV antigen-containing cells formed plaques, as visualized by immunocytochemistry (data not shown). Microglial cells, occasionally present in oligodendrocyte cultures, have never been found to contain viral antigen.

Fig. 3 shows the kinetics of virus production in neural cells and fibroblasts at an m.o.i. of 1. Astrocytes, oligodendrocytes and rat fibroblasts allowed replication of PRV at the same rate, whereas neurons showed a delayed rate of viral replication and 10- to 100-fold lower yields of virus during the first 20 h post-infection.

Antiviral effects of cytokines in neural cells

The antiviral activities of the cytokines tested in different neural cells and fibroblasts infected at a low m.o.i. (0.0005) are shown in Fig. 2. Both IFN- γ and IFN- β effectively inhibited the formation of antigen-positive plaques in all neural cell types as well as in fibroblasts. On the other hand, TNF- α treatment inhibited plaque formation to a moderate extent only in astrocyte cultures (Fig. 2*b*). Exposure of cells to combinations of IFN- γ and TNF- α revealed a modest synergistic effect in neurons,

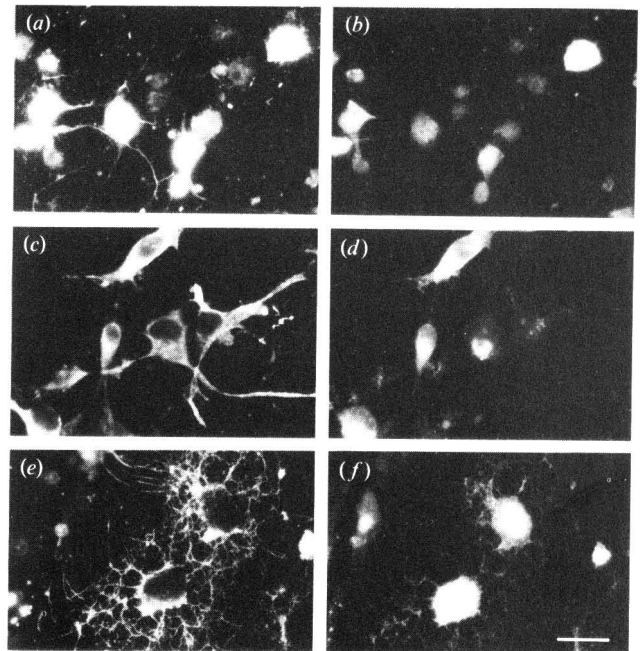


Fig. 1. Identification of PRV-positive neural cell types with markers specific for viral antigen. Double immunofluorescence labelling shows PRV-positive staining (*b*, *d* and *f*) in NF⁺ neuronal (*a*), GFAP⁺ astroglial (*c*) and GALC⁺ oligodendroglial cells (*e*). Bar marker represents 16 μ m.

oligodendrocytes and fibroblasts, whereas in astrocytes the pronounced inhibition exerted by IFN- γ alone was not enhanced by TNF- α .

Since the antiviral activity of IFNs is known to be challenge dose-dependent, we also tested the cytokines at an m.o.i. of 1.0. The virus yield reduction assays (Fig. 3) revealed that IFN- β is highly effective in astrocytes and fibroblast cultures and to a lesser extent in neuronal and oligodendroglial cells. IFN- γ treatment resulted in an approximately 10-fold reduction in virus yields from both glial cell types (Fig. 3*b,c*) and fibroblasts (Fig. 3*d*), whereas the yield of infectious virus from neurons was less affected (Fig. 3*a*). TNF- α treatment alone did not affect the virus yields nor did it exhibit clear synergism with IFN- γ in all cell types tested, except for fibroblasts (Fig. 3*d*).

Effect of cytokines on viral protein synthesis

The data on viral protein synthesis in neural cells and fibroblasts exposed to the different cytokines are shown in Fig. 4. Inhibition of viral protein synthesis correlated well with the inhibition of virus yields in most cell types. PAGE analysis showed that synthesis of the major viral proteins of PRV occurred in cytokine-exposed neurons and oligodendrocytes (not shown). In contrast, IFN- β

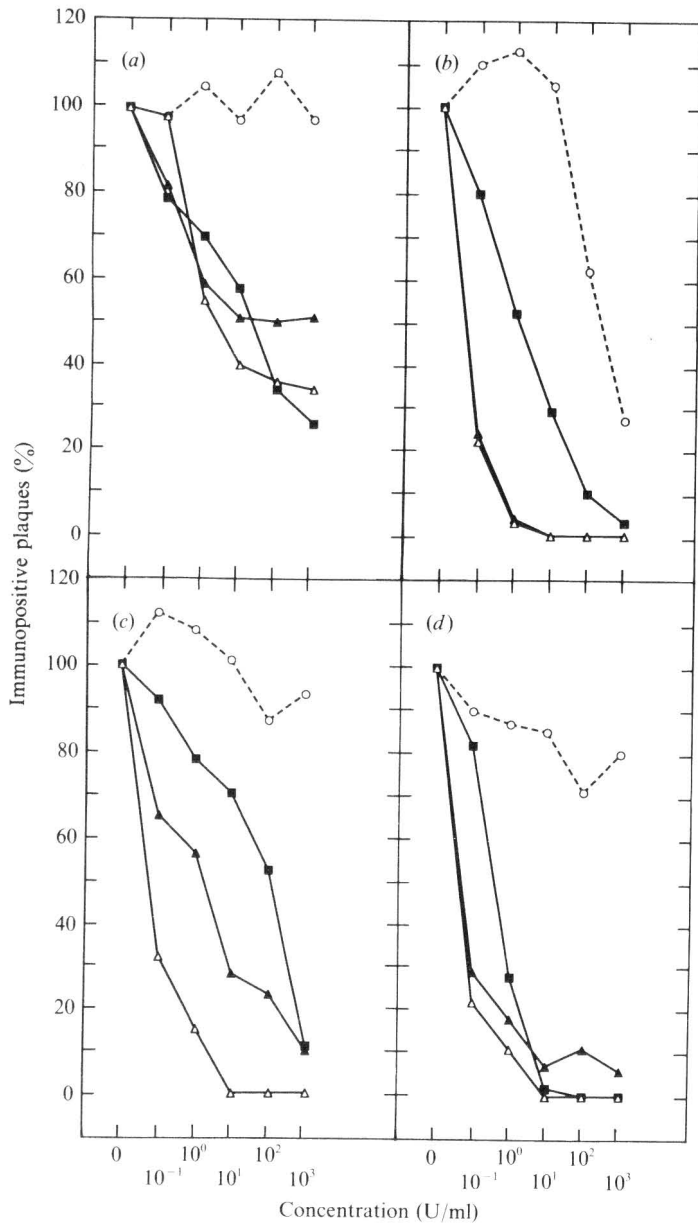


Fig. 2. Inhibitory effects of cytokines on PRV immunopositive plaque formation. Neurons (a), astrocytes (b), oligodendrocytes (c), and fibroblasts (d) were pretreated with different doses of TNF- α (○), IFN- γ (▲), IFN- β (■), and combinations of TNF- α and IFN- γ (△), before infection with 500 p.f.u. PRV/well. At 24 h after infection, plaque formation was visualized by immunocytochemistry. The number of immunopositive plaques is expressed as a percentage of the control values.

effectively inhibited viral protein synthesis in astrocytes and fibroblasts (Fig. 4). IFN- γ inhibited viral protein synthesis as effectively as IFN- β in astrocytes, whereas in neurons and oligodendrocytes synthesis of viral proteins did occur (data not shown) in spite of an evident inhibition of virus yield (Fig. 3). In fibroblasts, IFN- γ

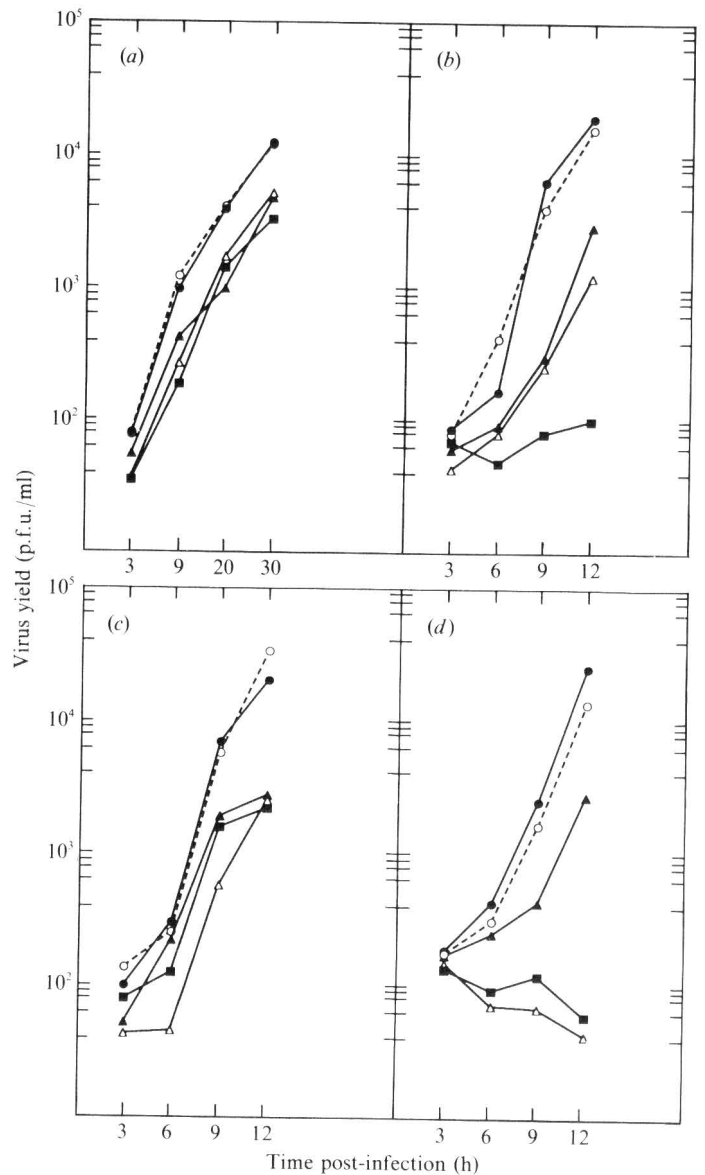


Fig. 3. PRV yields at various times after infection in cytokine-treated neurons (a), astrocytes (b), oligodendrocytes (c), and fibroblasts (d). Cells were exposed for 24 h to TNF- α (○), IFN- γ (▲), IFN- β (■), or combinations of TNF- α and IFN- γ (△), or were untreated (●), before infection at an m.o.i. of 1.

inhibited synthesis of some viral proteins (Fig. 4). In agreement with these findings we observed a decrease in the number of antigen-containing cells in cytokine-treated monolayers (not shown).

Discussion

To evaluate the effects of cytokines in neural virus infection we tested the individual antiviral activities of TNF- α , IFN- γ and IFN- β , and of combinations of IFN- γ

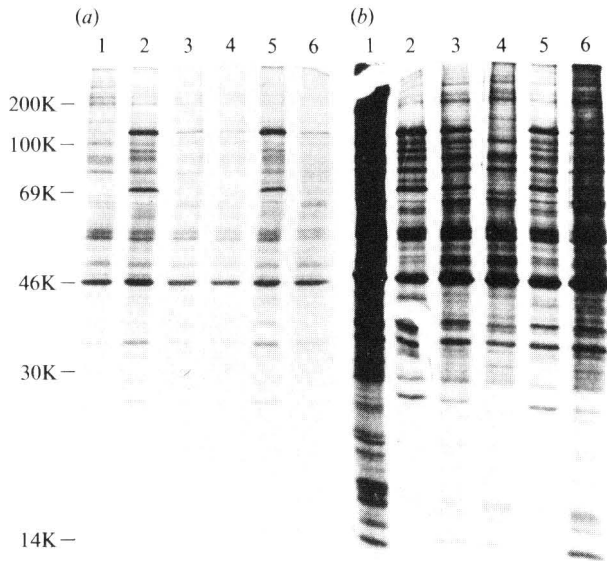


Fig. 4. Effect of cytokines on PRV protein synthesis in astrocytes (a) and fibroblasts (b). Cells were untreated (lanes 2) or exposed to TNF- α (lanes 5) (10^2 U/ml), IFN- γ (lanes 3) (10^3 U/ml), IFN- β (lanes 4) (10^3 U/ml), or a combination of TNF- α (10^2 U/ml) and IFN- γ (10^3 U/ml) (lanes 6), and infected with PRV at an m.o.i. of 1, or were mock-infected. Cells were labelled with [35 S]methionine for 1 h at 9 h after infection. Proteins were analysed by SDS-PAGE, followed by autoradiography. Lanes 1 show a cell control; M_r s are indicated to the left of (a).

and TNF- α . Since the extent of antiviral activity of cytokines is cell type-specific it is important to select the proper cell-cytokine combination for study. We used primary cultures enriched for neurons, astrocytes or oligodendrocytes as representatives of the major cell types of the CNS. All cell types allowed replication of PRV, but neurons showed a relatively low degree of permissiveness. Together with observations *in vivo*, this finding suggests that (in addition to neuronal spread) infection of glial cells may represent an important pathogenetic mechanism, providing large quantities of extracellular virus for infection of surrounding neural cells. Microglial cells, occasionally present in oligodendrocyte-enriched glial cultures, have never been found to contain viral antigen. In view of their macrophage-like properties (Frei *et al.*, 1986) and the known non-permissiveness of peritoneal and peripheral blood macrophages (Schijns *et al.*, 1989), it is unlikely that microglial cells allow PRV replication.

Intracerebral clearance of virus by T cells as well as production of IFNs and other cytokines during viral meningitis and encephalitis have been demonstrated in patients and animal models (Oldstone *et al.*, 1986; Sussman *et al.*, 1989; Frei *et al.*, 1988; Lebon *et al.*, 1989).

However, little is known about the antiviral properties of cytokines, especially IFN- γ and TNF, in neural cells. Previous studies have shown that IFN- β and human IFN- α A/D can inhibit the replication of HSV in rat and human neuron cultures, respectively (Svennerholm *et al.*, 1989; Pulliam *et al.*, 1986). Neurons produce small amounts of IFN only upon viral infection (Tedeschi *et al.*, 1986) and are probably largely dependent on the release of IFN by adjacent glial elements or infiltrating immune cells. Our results show that neurons are sensitive to the antiviral activity of IFN- γ and IFN- β and to the synergistic effect of combined exposure to IFN- γ and TNF- α . Interestingly, TNF- α treatment alone did not affect virus activity in neurons. However, when compared to glial elements neurons are less sensitive to the antiviral activity of cytokines, a characteristic which may predispose them to herpesvirus latency.

Among the neural cells, astrocytes are most sensitive to the antiviral activity of IFN- γ and TNF- α . Their sensitivity to IFN- γ has also been reported for other functions which illustrates the immunoregulatory role of this cell type. Astrocytes produce interleukin-1 (IL-1), IL-3, IL-6, TNF and IFN- α/β (Lieberman *et al.*, 1989; Frei *et al.*, 1986), they show MHC-restricted antigen presenting capacity for T cells, they express adhesion molecules (Frohman *et al.*, 1989) and are, together with microglial cells, the brain cells which express MHC class II antigens. Most of these functions are induced or stimulated by IFN- γ (Fierz & Fontana, 1986). The various interactions between astrocytes and IFN- γ and TNF released by recruited leukocytes may therefore play a key role in inhibiting virus spread to surrounding neural tissues. The high sensitivity of astrocytes to IFN- γ is also reflected in the efficient inhibition of viral protein synthesis. The identical protein patterns in protected astrocytes show that both IFN- γ and IFN- β block the virus replication cycle at a similar stage, before or during viral protein synthesis. In contrast, we found that protein synthesis was unimpaired in cytokine-exposed neurons, although assays of parallel cultures revealed a reduction in infectivity yield. In view of the yield reduction assays we feel that these proteins are derived from non-protected, virus-replicating cells and not from interferon-protected cells in which the virus was inhibited at a late post-transcriptional stage.

A complicating factor is the observation that the antiviral activity of IFNs depends upon the challenge dose (Stitz & Schellekens, 1980). As expected, inoculation of virus at higher m.o.i. partly compensated for the inhibitory effects of IFN- γ and TNF- α , whereas IFN- β activity remained pronounced in astrocytes and fibroblasts. However, herpesvirus infections *in vivo* are characterized by cell-to-cell spread and are therefore most realistically reflected by inoculation at low m.o.i. *in*

vitro. When taking into account that herpesviruses are relatively IFN-resistant, our results show an antiviral activity of TNF- α , IFN- γ and IFN- β in neural cells. This observation implies that immune cell- or brain cell-derived cytokines may be instrumental in immunity against cerebral virus infections, not only by acting as immunoregulatory molecules but also by directly interfering in virus replication. Furthermore, local cytokine therapy may be beneficial; our recent findings have shown that IFN- γ administered into the ventricular system of the brain protects rats against the fatal outcome of intracerebral and peripheral PRV infection (Schijns *et al.*, 1990).

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