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Cytokine production in the nervous system of mice during acute and latent infection with herpes simplex virus type 1

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Immunocytochemistry on serial paraffin sections was used to monitor the production dynamics of cytokines (IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α) and viral antigens in the trigeminal ganglion (TG) and the central side of the dorsal root entry zone (DRE) of mice, following infection of the cornea with herpes simplex virus type 1. In normal TG, scattered satellite cells were TNF-α and in the DRE, TNF-α and/or low numbers of IL-6 cells were detected. On day 3 after infection, foci of TG neurons with viral antigens were surrounded by large numbers of TNF-α and/or IL-6 cells and low numbers of IFN-γ cells. IL-2 and/or IL-4 cells appeared later, when viral antigens had almost cleared. In the TG, the most striking changes occurred with TNF-α, with respect to its source (satellite cells, Schwann cells and infiltrating cells) and the extent and long duration of its production. TNF-α was the predominant cytokine throughout acute and latent infection and even by day 30, numbers of satellite cells expressing this cytokine were three times higher than those in normal ganglia. Moreover, in the DRE, TNF-α was the only cytokine detected during virus clearance and again, its production continued, along with that of IL-6, on days 20 to 30, in both infiltrating cells and astrocytes. Thus, cytokines, particularly TNF-α and perhaps IL-6, from infiltrating cells and resident glial cells may have a role both in virus clearance and in normal homeostatic mechanisms in the nervous system such as repair and protection of neurons from damage.

Introduction

Interactions between herpes simplex virus type 1 (HSV-1) and sensory ganglia are pivotal to the pathogenesis of virus infection. This tissue can support active virus replication and neurons within it constitute the major site of latency. The immune system is one of the dominant elements controlling virus–ganglion interactions in acute (Shimeld *et al.*, 1995; Cantin *et al.*, 1995; Liu *et al.*, 1996) and reactivated infection (Shimeld *et al.*, 1996), but many aspects of the underlying mechanisms are poorly understood. Its role in the maintenance and establishment of latency is less clear.

Following infection of the cornea, virus can spread to the central nervous system (CNS) in addition to replication in the trigeminal ganglion (TG). HSV-1 has been isolated from the brain stem after inoculation of the cornea (Tullo *et al.*, 1982) and viral antigens have been demonstrated in the central side of the dorsal root entry zone (DRE) (Shimeld *et al.*, 1995). The immune response at this site is largely unexplored and it may differ from that in the ganglion because of the blood–brain barrier and the different glial cells in the CNS. Responses at this site are also of particular interest in view of the demyelination which has been regularly reported on the CNS side of the junction and the likely role of immunopathology in this damage (Hill, 1983).

Recently, we have investigated the phenotype of immune cells which infiltrate the TG during primary infection with HSV-1 in the mouse (Shimeld *et al.*, 1995). This study showed that, during virus clearance, there was an increase in the numbers of cells belonging to the macrophage/dendritic cell lineage and increased expression of major histocompatibility complex (MHC) class II antigens, including *de novo* expression on satellite and Schwann cells in areas where viral antigens were detected. This may allow early presentation of antigens even though only low numbers of $CD4^+$ or $CD8^+$ T cells were present at this time. After virus clearance, there was a second large peak of MHC class II expression together with increasing numbers of T and B cells. Large levels of such expression, together with large numbers of these lymphocytes, persisted in the TG well into latency. A similar timing of infiltration and persistence of $CD4^+$ and $CD8^+$ T cells has been reported

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previously (Cantin *et al.*, 1995) but there is conflicting evidence as to which of these cell types predominate (Shimeld *et al.*, 1995; Liu *et al.*, 1996).

There has been limited characterization of the immune response in the TG by immunocytochemical detection of cytokines. In one study, IFN-γ, IL-4, IL-10 and TNF were investigated (Liu *et al.*, 1996), and in the other, IFN-γ alone was examined (Cantin *et al.*, 1995). In our study, the inclusion of the TG with its attached DRE has facilitated, for the first time, a simultaneous comparison between events in the ganglion and the CNS. Moreover, we have used a more extensive range of cytokines and have investigated the spatial relationship between cytokine-producing cells and those expressing HSV-1 antigens. This latter aim was made possible by the recent development of a technique using paraffin-embedded tissue, which provides both excellent tissue morphology and reliable detection of cytokines (J. L. Whiteland, C. Shimeld, S. M. Nicholls, D. L. Easty, N. A. Williams & T. J. Hill, unpublished results). We now report the dynamics of production of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α and viral antigens in the TG and DRE during infection with HSV-1, in order to provide further information on the possible role of these cytokines in clearance of virus and at much later times during latency.

Methods

■ **Mice.** Specific-pathogen-free, 8-week-old NIH/OLA inbred female mice were obtained from Harlan/Olac. They were maintained as a breeding colony in the School of Medical Sciences Animal House, Bristol, UK. Mice were anaesthetized as described previously (Shimeld *et al.*, 1995) and inoculated by scarification of the left cornea with a 26 gauge needle through a 5 μl drop of medium containing 1×10^4 p.f.u. of HSV-1 strain McKrae (Williams *et al.*, 1965). Control mice were inoculated in the same way with a preparation of uninfected Vero cells made in the same manner as the virus inoculum (mock inoculum).

■ **Dissection and processing of tissues.** Anaesthetized mice were perfused with periodate–lysine–phosphate buffer (PLP) (Whiteland *et al.*, 1995). The left TG with its DRE attached was carefully dissected from the skull and placed ventral surface down in curetting cassettes. Other tissues (eyes, small intestine and spleen) were taken as positive controls for staining for cytokines. Five days after infection, the inoculated eye was removed. For eyes to be fixed in PLP, two holes were made in the back with a 30 gauge needle; PLP was gently injected through one of these until fixative flowed out of the other. From normal mice, the small intestine was removed and 4 to 5 mm pieces containing Peyer's patches were cut; spleens were also removed and cut into 2 mm slices.

Tissues were processed as described previously (Whiteland *et al.*, 1995). In brief, they were fixed overnight in PLP at 4 °C, rapidly dehydrated and infiltrated under vacuum with low temperature paraffin wax. Serial 6 μm sections were cut and transferred to glass microscope slides precoated with poly-L-lysine. Slides were dried overnight at 37 °C, wrapped in aluminium foil and stored desiccated at −20 °C.

■ **Immunohistochemistry**

(i) Staining for cytokines. The method was adapted from that of Andersson *et al.* (1994). Slides were defrosted and dried at 37 °C for 30 min, dewaxed in Histoclear (3 × 5 min) and rehydrated in 100% ethanol (2 × 5 min), 75% ethanol (5 min) and PBS containing 0·1% saponin (Sigma) (PBS-S) (2 × 5 min). Excess PBS-S was removed and the sections were circled with a water-resistant pen (Dako). Non-specific binding sites were blocked by incubation for 30 min at room temperature with 1·5% normal rabbit serum (NRS) diluted in PBS-S with 2% BSA (2% PBSA-S), except for sections to be stained for IL-2 which were incubated with 5% NRS diluted in 0·1% PBSA-S. Sections were incubated with primary antibody overnight at 4 °C. The clones and concentrations of the rat anti-mouse monoclonal antibodies used for staining were as follows: anti-IL-2 (SB46, 10 μg/ml); anti-IL-4 (BVD4-1D11, 50 μg/ml); anti-IL-6 (MP5-20F3, 50 μg/ml); anti-IL-10 (JES-2A5, 3·3 μg/ml); anti-IFN-γ (XMG1.2, 2·5 μg/ml); and anti-TNF-α (MPG-XT22, 20 μg/ml). In addition, the following rat isotype controls were used: IgG1 (R3-34), IgG2a (R35-95) and IgG2b (R35-38). Sections were then incubated with biotinylated rabbit anti-rat Igs diluted in 0·1% PBSA-S, followed by ABC–horseradish peroxidase complex diluted in PBS-S; both incubations were carried out for 30 min at room temperature. The chromogen was Vector VIP diluted in PBS only, which was used according to the Vectorstain protocol and gave a purple end-product. PBS-S (2 × 5 min) was used for all wash steps between incubations, except for the final wash step before the VIP reagent, which was with PBS only. The monoclonal antibody to IL-10 was obtained from Harlan Sera Lab, and all the others were from PharMingen. All blocking serum, secondary antibody, ABC Elite reagent and Vector VIP substrate were obtained from Vector Laboratories (Peterborough, UK). Sections were counterstained with methyl green, dehydrated, cleared and mounted in Histomount (National Diagnostics).

Positive and negative controls were included in each staining run. Positive control sections for cytokine staining were as follows: small intestine with Peyer's patch stained for IL-2, IL-4 and IL-10; spleen for TNF-α; and eyes, 5 days after inoculation of HSV-1, for IL-6 and IFN-γ. Observations have shown that such tissues are reliable sources of these cytokines. Comparative observations demonstrated that staining for these cytokines in PLP-fixed, paraffin-embedded tissue was equivalent to or better than that in formaldehyde-fixed frozen tissue (J. L. Whiteland, C. Shimeld, S. M. Nicholls, D. L. Easty, N. A. Williams & T. J. Hill, unpublished results). Sections of HSV-infected eyes were also used as positive controls for staining for HSV antigens. To check the specificity of staining, negative control slides were prepared of sections of TG/DRE incubated with diluent instead of primary antibody, or with rat IgG1, IgG2a or IgG2b isotype controls appropriate to the isotype of, and at the same concentration as, the respective monoclonal antibody against the cytokine.

(ii) Double staining for cytokines and HSV-1 antigens. Sections were first stained for cytokines as described above except that, following application of VIP, sections were washed twice in PBS. They were then stained for HSV-1 antigens by the peroxidase–anti-peroxidase method at room temperature using, in sequence, 10% normal swine serum, rabbit anti-HSV serum diluted 1:500 in PBS containing 0·1% BSA, swine anti-rabbit Ig diluted 1:100 in PBS and rabbit peroxidase–anti-peroxidase complex diluted 1:100 in PBS (all sera were from Dako). The chromogen was diaminobenzidine (Sigma) which gives a brown end-product. Slides were incubated at room temperature for 2 h with the primary antibody and for 30 min with the other antibodies. Sections were washed twice in PBS between steps.

■ **Identification and quantification of stained cells.** Cytokine-producing cells were identified by the presence of intracellular immunoreactivity using × 40 or × 100 objectives. Extracellular staining in the absence of a co-producer cell was disregarded. Assessment for each anti-cytokine antibody was done in the area of maximum staining. The

number of positive cells in whole TG and in the CNS side of the DRE were counted in three sections from each of the samples. At some time-points, after staining for TNF-α or IL-6, the number and density of stained cells was so high that it was impossible to count cells accurately; such samples were designated to have > 200 stained cells. The mean area of TG was $4{\cdot}2 \times 10^6$ μm² and that of the CNS was $5{\cdot}8 \times 10^5$ μm².

Results

Experimental protocol

On each of days 3, 5, 7, 10, 20 and 30 after inoculation of the cornea, seven to eleven mice inoculated with virus and two to four mice given mock inoculum were killed and the TG/DRE removed. Similar samples were removed from two normal mice to identify any cytokine-producing cells resident in these tissues. Samples were processed for immunohistochemistry and serial sections cut. The majority of sections were double-stained for cytokines and HSV-1 antigens; the remainder were stained for cytokines alone. Serial sections of two TG/DRE from mice inoculated with virus 14 days previously were stained for cells producing IL-2 and/or IL-4 alone. In total, approximately 20000 sections were stained and examined.

Nature of staining for cytokines in TG/DRE and in control tissues

Three different types of cellular staining for cytokines were observed. With all the cytokine antibodies, positive staining of cells with small intracytoplasmic granules was observed (Fig. 1*e*, *f*). Some cells which stained for IL-2, IL-4 or IFN-γ showed cytoplasmic staining with extracellular staining surrounding the producer cell (Fig. 1*h*). With antibodies to TNF-α and IL-6, more generalized cytoplasmic staining was seen (Fig. 1*i*, *j*, *k*, *l*).

Negative control sections of TG/DRE incubated with diluent instead of primary antibody or with isotype control antibodies showed no background or non-specific staining.

Cytokines in TG/DRE from normal and mock-inoculated mice

TNF-α expression was detected on scattered cells which were evenly distributed throughout the TG (Fig. 1*a*); cell morphology and location suggested strongly that they were satellite cells (Fig. 1*b*). On this basis, such cells will henceforth be referred to as satellite cells; definitive identification of these cells was not possible since there is no reliable antigenic marker for satellite cells. The satellite cells associated with approximately 30% of neurons were TNF-α^+. Positive staining for TNF-α was also seen in cells with dendritic morphology that lay in a line in the CNS at the DRE (Fig. 1*c*). A lower number of such cells also showed faint staining for IL-6. No other cytokines were detected. Similar numbers, types and distribution of stained cells were found in samples from mock-inoculated mice.

Cytokines and HSV-1 antigens in TG from infected mice

The numbers, types and distribution of cells which stained for viral antigen were similar to those described previously (Shimeld *et al.*, 1995). In brief, antigen was present in the TG from days 3 to 10 after inoculation of virus. It was first seen in the dorsal part of TG1 (ophthalmic part of the TG) and by day 5, was present throughout all parts of the ganglion. In one TG sample taken on day 30, a single neuron stained for HSV-1 antigens; it was located in the rows of neuronal cell bodies that lie at the medial edge of TG1. No HSV-1 antigens were detected in samples from mock-inoculated mice.

Early in infection (day 3), foci of neurons and satellite cells which stained for HSV-1 antigens contained numerous TNF-α^+ (Fig. 1*d*) and/or IL-6^+ (Fig. 1*e*) infiltrating cells and lower numbers of such cells which were IFN-γ^+ (Fig. 1*f*, Table 1). In and around these foci, all satellite cells stained for TNF and some stained for IL-6. No staining for IFN-γ and/or IL-6 was seen in areas of TG distant from HSV-1-infected foci. In contrast, there was a large increase in the number of satellite cells that stained for TNF-α throughout the entire ganglion, although staining of such cells was more frequent and stronger in infected areas. When staining for viral antigen was maximal (day 5), the numbers of cells which stained for TNF-α remained high whereas the numbers of IL-6^+ and/or IFN-γ^+ cells declined.

When viral antigens were almost cleared (days 7 to 10) the level of TNF-α expression increased dramatically. There were three major sources of this cytokine: (i) large numbers of infiltrating cells; (ii) all satellite cells within the area of maximum staining and even the majority of these cells in the rest of the TG; and (iii) cells, particularly in TG1, which by their distribution and morphology were probably Schwann cells. On day 10, there was a modest increase in the numbers of IFN-γ^+ cells and a very low number of cells which stained for IL-2.

Only after clearance of virus (day 14) were appreciable numbers of IL-2- or IL-4-producing cells detected; numbers of these cells continued to rise well into latency (day 20) (Fig. 1*g*, *h*). They were most densely distributed in TG1 but were also scattered throughout all three parts of the TG, including between axons in the peripheral nervous system (PNS) close to the DRE. Also at this time, there was a second large peak of IL-6 expression both in a large number of satellite cells and in dendritic processes of immune cells (Fig. 1*i*). This expression occurred throughout the entire ganglion but was most concentrated in the dorsal part of TG1. By day 30, the number of cells which stained for IL-2, IL-4 and IL-6 had declined substantially. However, TNF-α expression remained far (three-fold) higher than that seen in TG from normal or mock-inoculated mice (Fig. 1*j*). No cells producing IL-10 were detected at any time-point tested.

The neuron obtained from one of the ten TG taken on day 30 which stained for viral antigen and is presumably the result of spontaneous reactivation, was found on sections that stained

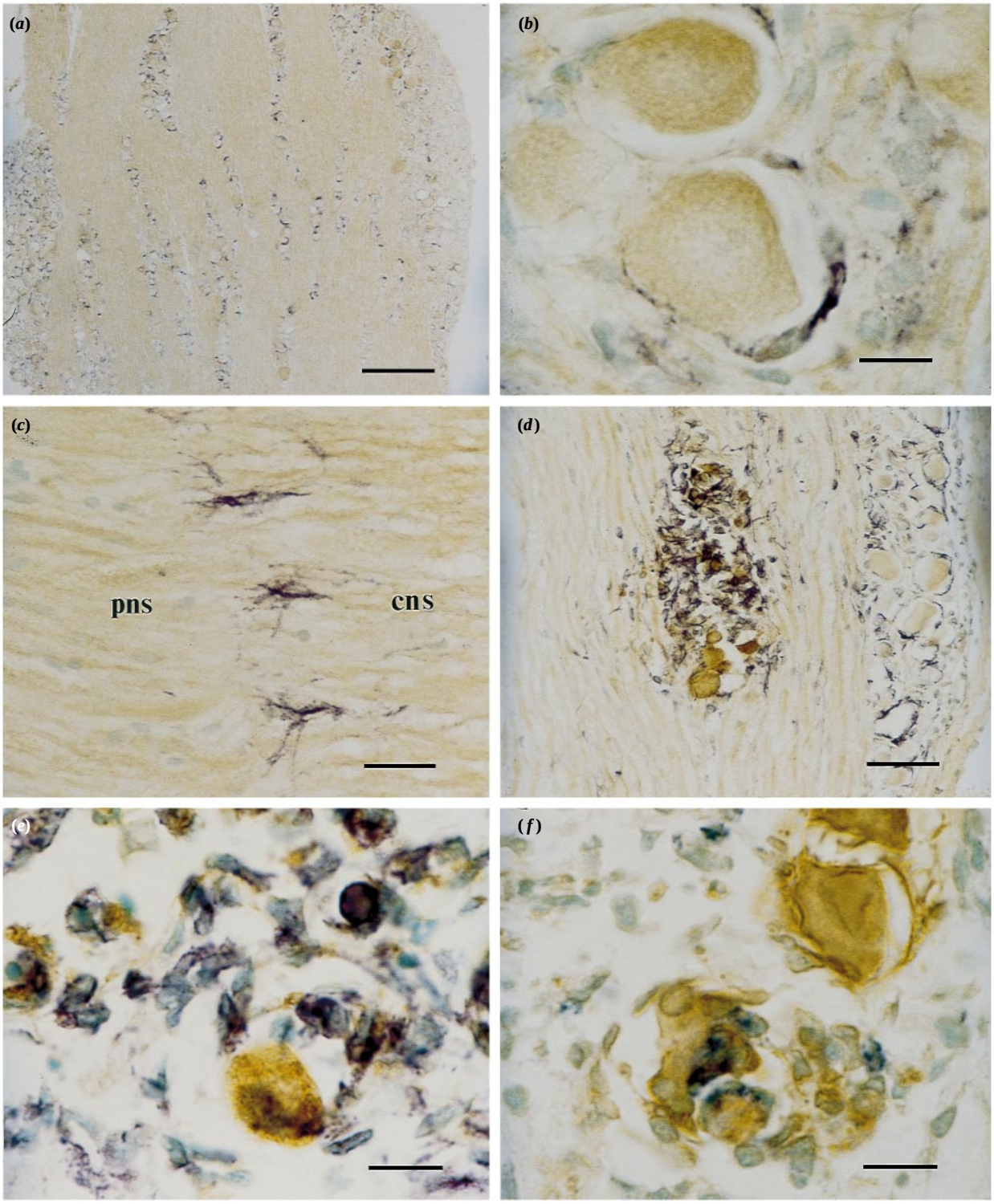
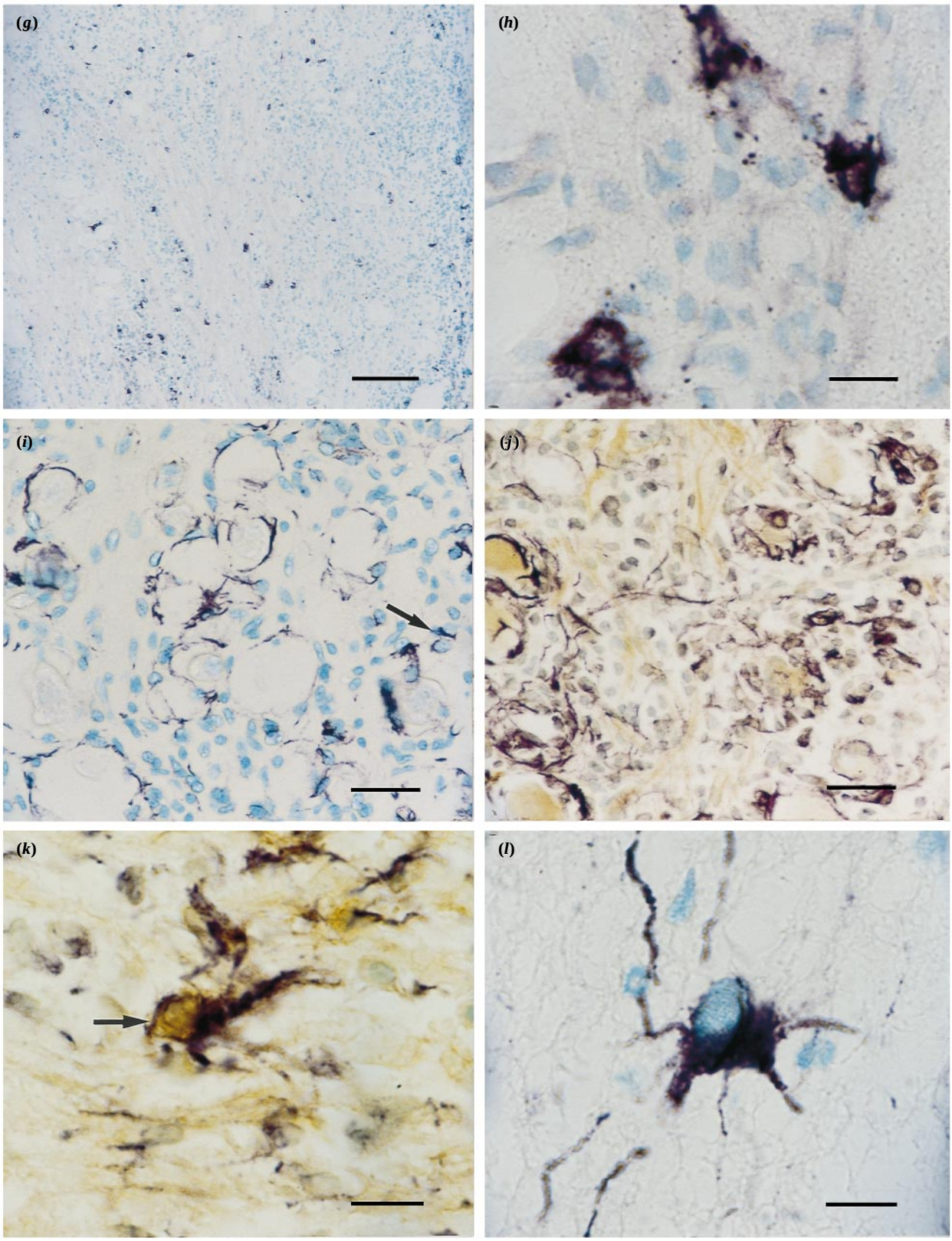


Fig. 1. Immunohistochemical detection of HSV-1 antigens (brown) and cytokines (purple) (*a*, *b*, *c*, *d*, *e*, *f*, *j*, *k*) or cytokines alone (*g*, *h*, *i*, *l*) in TG and DRE. Samples from normal mice: (*a*) TG with scattered TNFα^+ satellite cells; (*b*) higher magnification of (*a*); and (*c*) TNF-α^+ cells with dendritic morphology in the CNS at the DRE. Samples taken from mice after inoculation of virus on the cornea: TG, day 3, virus-infected cells, including neurons, surrounded by (*d*) TNF-α^+ infiltrate, (*e*) IL-6$^+$ infiltrate, and (*f*) a sparse IFN-γ^+ infiltrate; TG, day 20, (*g*) scattered IL-4$^+$ cells, (*h*) IL-2$^+$ cells and (*i*) IL-6$^+$ satellite cells and IL-6$^+$



dendritic processes (arrow); TG, day 30, (*j*) extensive expression of TNFα in satellite cells and infiltrate; DRE, day 5, (*k*) cell with viral antigens in cell body and TNFα in its dendritic processes (arrow) in the CNS; DRE, day 20, (*l*) IL-6^{+} astrocyte in the CNS. Bar represents 250 μm (*a*), 100 μm (*g*), 50 μm (*d*), 25 μm (*c*, *i*, *j*) and 10 μm (*b*, *e*, *f*, *h*, *l*, *k*).

Table 1. Numbers of cytokine-producing cells in the TG and DRE after infection of the mouse cornea with HSV-1

Numbers represent mean of counts ± SEM, in the area of maximum staining from three sections. Sections with more than 200 stained cells were impossible to count accurately. ND, Not done.

Days after inoculation	Cytokine					
	IL-2	IL-4	IL-6	IL-10	TNF-α	IFN-γ
In the TG						
−1	0	0	0	0	30 ± 5	0
3	0	0	180 ± 23	0	150 ± 25	38 ± 8
5	0	0	5 ± 0·5	0	103 ± 22	3 ± 0·4
7	0	0	10 ± 2	0	> 200†	5 ± 2
10	1 ± 0·2	0	21 ± 2	0	> 200	30 ± 6
14	40 ± 4	15 ± 3	ND	ND	ND	ND
20	75 ± 11	75 ± 22	> 200	0	110 ± 14	5 ± 2
30	3 ± 0·2	11 ± 2	10 ± 3	0	100 ± 26	11 ± 3
In the central side of the DRE						
−1	0	0	3 ± 1	0	10 ± 3	0
3	0	0	0	0	11 ± 2	0
5	0	0	0	0	74 ± 8	0
7	0	0	0	0	103 ± 5	0
10	0	0	1 ± 0·6	0	> 200	2 ± 0·2
14	0	0	ND	ND	ND	ND
20	2 ± 0·6	1 ± 0·3	30 ± 13	0	40 ± 6	0
30	0	0	27 ± 5	0	76 ± 8	1 ± 0·2

either for IFN-γ or for TNF-α. Low levels of staining for TNF-α were detected in infiltrating cells surrounding the neuron but no IFN-γ was seen.

Cytokines and HSV-1 antigens in DRE from infected mice

Viral antigens were present in the CNS side of the DRE from days 5 to 10 after inoculation of virus. An increase in the number of TNF-α^+ cells, which was about sevenfold higher than that seen in normal mice, coincided with the first appearance of viral antigens (Table 1). Two morphologically distinct types of cells expressed this cytokine; one was 'round' and the other had dendritic processes. Double staining was seen in some cells with dendritic morphology; the cell body stained for viral antigens and the dendritic processes for TNF-α (Fig. 1*k*). During days 7 to 10, when viral antigens were almost cleared, the numbers of TNF-α^+ cells increased to more than twenty times the normal level and on day 10 cells which by their morphology appeared to be astrocytes were also stained. Very low numbers of IL-6^+ cells were seen at this time.

During the latent period, TNF-α was not detected in 'round' cells but was still present in the cells with dendritic morphology and astrocytes. Increased numbers of cells which stained for IL-6 were seen on days 20 to 30. The morphology of these cells was similar to that of cells which stained for TNF-α, including those which appeared to be astrocytes (Fig. 1*l*). No IL-10^+ cells and only very low numbers of IL-2^+, IL-4^+ and/or IFN-γ^+ cells were detected.

Discussion

We believe this to be the first report of TNF-α production in the normal TG, although mRNA for this cytokine has previously been demonstrated in such tissue (Halford *et al.*, 1996); hence this pleiotropic cytokine may be involved in homeostasis in the PNS. It is therefore noteworthy that many neurons in the normal ganglion constitutively express TNF receptors (Cunningham *et al.*, 1997). A homeostatic function has been suggested for TNF-α from glial cells in the CNS (Breder *et al.*, 1993) and IL-1 from Schwann cells in the PNS (Bergsteinsdottir *et al.*, 1991).

Early cellular infiltration into foci of HSV-1 antigen in TG1 was accompanied by production of IFN-γ, IL-6 and TNF-α and an increase in the numbers of TNF-α^+ satellite cells in these foci. Production of TNF-α by glial cells in the TG has also been reported following intranasal inoculation of HSV-1 (Walev *et al.*, 1995). In the present study, the staining pattern of satellite cells was similar to the pattern of *de novo* MHC class II expression seen in these cells at similar times (Shimeld *et al.*, 1995). This similarity in staining pattern was even more striking on later days when large numbers of cells (most likely Schwann cells) were also stained. This pattern is perhaps not

surprising since the genes for TNF-α and MHC are subject to similar transcriptional control, in particular via IFN-γ (Billiau, 1996) or TNF-α (Panek *et al.*, 1992). An IFN-γ^+ infiltrate developed in infected foci in the TG on day 3 and was present, albeit in lesser amounts, throughout the period of study. Besides affecting the production of other cytokines, IFN-γ may directly mediate virus clearance (Raniero de Stasio *et al.*, 1990). Whether IL-6, which is also present early in infection, has antiviral activity is controversial (Akira *et al.*, 1993). Macrophages and dendritic cells infiltrate the TG early in infection (Shimeld *et al.*, 1995; Liu *et al.*, 1996) and double staining for cytokines and cell surface molecules showed that some of the TNF-α^+ and/or IL-6^+ cells were such infiltrates (data not shown). Low numbers of granulocytes have also been identified in HSV-1-infected foci in ganglia (Shimeld *et al.*, 1995) and these can produce TNF-α (Bazzoni *et al.*, 1991) and possibly IFN-γ (J. L. Whiteland, C. Shimeld, S. M. Nicholls, D. L. Easty, N. A. Williams & T. J. Hill, unpublished results). NK cells may also form part of the early infiltrate (Liu *et al.*, 1996) and these cells are potent producers of IFN-γ.

After the initial response, the numbers of IFN$^+$ and/or IL-6^+ cells decline, whereas numbers of TNF-α^+ cells rise to even higher levels. Such TNF-α^+ cells include an extensive infiltrate and a large proportion of the TG glial cells. Indeed, many HSV-1-antigen-positive neurons were swathed in TNF-α^+ satellite cells. Thus, TNF-α is the predominant cytokine present in the TG, often in very close proximity to virus-infected cells, during the crucial times when HSV-1 is being cleared. TNF-α may limit virus replication in neurons either by inducing apoptosis or in other ways. For example, *in vivo*, TNF-α decreases hepatitis B virus gene expression (Gilles *et al.*, 1992), protects against infection with HSV-1 (Rossol-Voth *et al.*, 1991) and *in vitro* (in combination with IFN-γ), it blocks an early step in HSV-1 gene expression (Feduchi *et al.*, 1989).

CD8^+ T cells appear to be essential for clearance of HSV-1 from the TG (Simmons & Tscharke, 1992) and such cells increase in the ganglion during virus clearance (Shimeld *et al.*, 1995; Liu *et al.*, 1996). Antiviral cytokines from these cells may also play a role. For example, secretion of IFN-γ and TNF-α by CD8^+ cells following antigen recognition can abolish hepatitis B virus gene expression and replication (Guidotti & Chisari, 1996). In the present study, there was a substantial increase in TNF-α^+ cells and a modest increase in IFN-γ^+ cells at times when CD8^+ T cells were infiltrating the TG; it has been suggested that such cells may be a source of TNF-α (Liu *et al.*, 1996).

Large numbers of CD4^+ T cells also infiltrate the TG during virus clearance; however, very few IL-2-producing cells and no IL-4-producing cells were detected. Other groups have detected IL-4^+ cells as early as day 5 after inoculation (Liu *et al.*, 1996) and mRNA for IL-2 (Halford *et al.*, 1996). These differences may reflect the use of different mouse/virus strain combinations or differences between detection of mRNA and its protein product. For example, mRNA for TNF-α (Stevens, 1995) and for other cytokines (Feldmann *et al.*, 1991) may accumulate in cells without production of the protein.

TNF-α was the only cytokine detected in the DRE early in infection, thus underlining, as in the TG, its likely importance in virus clearance. At late times, when viral antigen had been cleared, in contrast to the situation in the TG, only low levels of IL-2 and IL-4 were detected in the DRE. However, high levels of TNF-α and IL-6 persisted in astrocytes and other cells. Astrocytes produce TNF-α under a variety of circumstances, including virus infection (Joseph *et al.*, 1993; Sun *et al.*, 1995). It is noteworthy that production of these potent macrophage activators occurs at the site where such activation may play a key role in the demyelination associated with HSV-1 infection in the CNS (Townsend, 1981). Indeed, TNF-α itself may have a direct involvement in demyelination through its toxicity for oligodendrocytes (Stoll *et al.*, 1993).

In contrast with other studies (Liu *et al.*, 1996), we failed to detect IL-10^+ cells in the TG, although such cells were always present in positive control tissue. Such differences may relate to the strain of mouse studied since there are large differences in the production of IL-10 in different rat strains (Issazadeh *et al.*, 1996). The absence of IL-10 in our studies is consistent with the observed persistence of TNF-α and MHC class II expression (Shimeld *et al.*, 1995) since IL-10 downregulates these proteins (Mossman, 1994).

The presence of a large number of immunologically active cells at late times, when production of viral antigens seems to have long ceased, raises a number of possibilities (Shimeld *et al.*, 1995; Halford *et al.*, 1996). One possibility is the presence of an unidentified HSV-1 antigenic stimulus, e.g. long-term presentation by antigen presenting cells, possible proteins encoded by LAT *in vivo* or products from low levels of ICP4 or TK mRNA (Kramer & Coen, 1995). Such persistence of HSV-1 antigens could account for the continued production of IL-2 and IL-4 which could produce clonal expansion of antigen-specific T cells and the stimulation/differentiation of B cells, respectively. Another possibility is the production of HSV-1 antigen in neurons following reactivation of latent infection. However, previous studies have shown that spontaneous reactivation in the mouse is a rare event (Tullo *et al.*, 1982; Willey *et al.*, 1984; Shimeld *et al.*, 1990) and, even in induced reactivation (Sawtell & Thompson, 1992; Shimeld *et al.*, 1996), only 1 to 3 neurons per ganglion were found to reactivate. In the present study, only one spontaneously reactivating neuron was detected in serial sections obtained from a total of ten TG taken at day 30. Given the rarity of this process, it seems unlikely that reactivation is responsible for the persistence of immunologically active cells in the TG during the period of latency. A further possibility is exposure of self-antigens during HSV-1 infection. There is evidence for transient immune response to myelin basic protein (a self-antigen in the CNS) during HSV-1 infection in the mouse (Bishop & Hill, 1989) and similar responses might occur to antigens of the PNS. Secretion of immunologically active cytokines from ganglionic cells,

including neurons, perhaps in response to tissue damage may be another possibility. Although such secretion from neurons, particularly in the CNS, has been reported (Bartfai & Schultzberg, 1993), no cytokine-positive sensory neurons were observed in this study. However, various glial cells can produce a number cytokines particularly in the CNS (Hopkins & Rothwell, 1995). In the present study, glial cells in the TG and CNS were clearly potent producers of TNF-α and IL-6, from early to late times after infection and covering the period when T and B cells persisted in the ganglion. Continued production of TNF-α could aid such persistence by increasing the permeability of endothelial cells and by inducing the expression of surface adhesion molecules.

Irrespective of the effects of neuronal or glial cell-derived cytokines on the persistence of immune cells in the TG, the late production of TNF-α and IL-6 suggest that their prime function may involve repair or another homeostatic mechanism in this tissue and in the CNS side of the DRE. Hence, with respect to IL-6, there was a dramatic increase in the number of satellite cells producing this cytokine on day 20. Although fibroblasts and astrocytes have been reported to produce IL-6 (Akira *et al*., 1993), to our knowledge, its production by satellite cells has not been reported before. The observation raises the possibility that IL-6 may be involved in the support and survival of sensory neurons; such a function has been described for this cytokine in relation to cholinergic neurons (Akira *et al*., 1993) and/or like IL-2 (Benveniste & Merrill, 1986), it may function in the repair of damaged tissue by acting as a growth factor for glial cells in the PNS. The most dramatic observations were made with TNF-α since even though cells with viral antigens were restricted to foci within the tissue, by late times (from days 14 to 30) when viral antigen was cleared, large numbers of satellite cells in the TG were producing this cytokine. Such a widespread response suggests an as yet undefined signalling mechanism between cells in the ganglion and a likely role for TNF-α in important homeostatic mechanisms in the PNS. Of particular interest in this respect is the recent observation that in the CNS, endogenous TNF-α from glial cells may play a major role in protecting neurons against excitotoxic and oxidative damage (Bruce *et al*., 1996). Hence, in addition to any antiviral activity of this cytokine, it may also protect neurons from damaging effects of the virus and thereby even play some part in virus latency.

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