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# Analysis of Cytokine mRNAs in Murine Herpes Simplex Virus Type 1 Retinitis

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**Purpose:** To investigate cytokine mRNA expression during the inflammatory process induced in the contralateral eyes by uniocular inoculation of herpes simplex virus type 1 (HSV-1) via the anterior chamber.

**Methods:** BALB/c mice were inoculated in the anterior chamber with $5 \times 10^4$ plaque-forming units of HSV-1 (KOS). mRNA was extracted from the inflamed posterior segments of the uninoculated eyes at 0 (control), 9, 11, 14, and 21 days postinoculation (p.i.). Reverse transcription-polymerase chain reaction was performed for semiquantitative analysis of mRNA expression of interleukin (IL)-1β, IL-2, IL-4, IL-10, IL-12p35, IL-12p40, interferon (IFN)γ, tumor necrosis factor (TNF)α, transforming growth factor (TGF)β2 and induced nitric oxide synthase (iNOS).

**Results:** Peak mRNA expression of iNOS was observed at day 14 p.i. The time profiles of mRNA expression for IL-1β, IL-2, IL-4, IL-10, IFN-γ, TNFα were similar to that of iNOS, while TGFβ2, IL-12p35, and IL-12p40 demonstrated a reverse pattern.

**Conclusions:** The kinetics of the analyzed cytokines synchronized with the clinicopathological activity of the experimental murine HSV-1 retinitis. The immunosuppressive cytokines TGFβ2 and IL-10 demonstrated different peaks of mRNA expressions suggesting that the down-regulation phase of the inflammatory process was controlled by several factors working at different phases. **Jpn J Ophthalmol 2003;47:166–172** © 2003 Japanese Ophthalmological Society

**Key Words:** Anterior chamber-associated immune deviation, cytokine, immunoregulation, retinitis, virus infection.

## Introduction

Uniocular inoculation of herpes simplex virus type 1 (HSV-1) into the anterior chamber (AC) of BALB/c mice induces retinitis in the contralateral, uninoculated eye, with sparing of the ipsilateral retina.[1] HSV-1 inoculated in the AC travels through the central nervous system, finally arriving at the contralateral sensory retina at day 7 postinoculation (p.i.), where virus replication occurs massively by day 10 p.i.[2] Clinical and histopathological examinations[3] could detect an early inflammatory process in the eye from day 8 p.i. (acute retinitis phase), followed by development of fulminant retinitis with complete destruction of normal intraocular tissue by day 14 p.i. (retinal necrosis phase), with gradual resolution of the retinitis (resolution phase). It has been presumed that numerous inflammatory factors account for the drastic inflammatory process following viral infection of the sensory retina, hence the clinicopathologic features. Immunologic studies[4,5] have revealed the importance of CD4 T cells in the modulation of HSV-1 retinitis and the effectivity of viral clearance. Recruitment of CD4 T cells into the contralateral eye was verified by immunohistochemical study and flow cytometric analysis. The exact function of CD4 T cells, however, still remains to be determined. Cytokines, mainly derived from inflammatory cells, including CD4 T cells, and sometimes from intraocular tissues, are known to play critical roles in inflammatory pro-

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cesses. We therefore evaluated the kinetics of several cytokines in experimental murine HSV-1 retinitis by employing a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) method.

## Materials and Methods

### *Animals*

Female BALB/c mice 6–12 weeks of age, purchased from Japan Clea Company (Suita, Osaka), were used in the experiments. All animal procedures were carried out in compliance with the Guidelines for Animal Research of Kobe University School of Medicine, and in accordance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research.

### *Virus*

The KOS strain of HSV-1, a kind gift from Dr. Sally Atherton, was used throughout these experiments. Virus stocks were propagated on vero cells and stored in aliquots at −80°C, as previously reported. The virus titer of the stock, determined by plaque forming assay, was $5 \times 10^8$ plaque-forming units (PFU)/mL.

### *RNA Isolation*

Samples were homogenized in Trizol reagent (GIBCO BRL, Grand Island, NY, USA) using a power homogenizer. Total RNA extracted from the samples was isolated by phenol and chloroform: isoamyl alcohol purification and by ethanol precipitation. The RNA pellet was dissolved in RNase-free water. The amount of the purified RNA was determined by spectrophotometry (OD 260/280 ratio $> 1.9$) and visualized by electrophoresis on a 1.0% ethidium bromide-stained agarose gel.

### *Reverse Transcription*

Using a commercially available kit (1st Strand cDNA Synthesis Kit for RT-PCR; Boehringer Mannheim, Germany), total RNA (0.1 μg) was reverse-transcribed (RT) into single-strand cDNA according to the manufacturer's protocol. The reaction mixture was incubated at 30°C for 10 minutes and then at 42°C for 60 minutes. Each cDNA sample was diluted in RNase-free water (1:10).

### *Calibration of Total RNA Amount*

The expression level of β-actin mRNA was examined separately. RT was performed as above. The primers used for β-actin PCR amplification are described in Table 1.

One-microliter aliquots of the RT-mix from each sample were added to the PCR master mix consisting of 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.8 mM dNTP, and 1.25 units of AmpliTaq Gold polymerase/reaction (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ, USA). Each β-actin primer (0.2 μM) was added to a final volume of 25 μL. The PCR cycle was as follows: preincubation at 94°C for 10 minutes, 28 cycles at 94°C, 50°C and 72°C for 1 minute each, and a final elongation step at 72°C for 15 minutes.

### *Semiquantitative RT-PCR*

To detect the kinetics of inflammatory cytokine mRNAs and induced nitric oxide synthase (iNOS) mRNA in the inflammatory process of HSV-1 retinitis, semiquantitative RT-PCR was performed. Total RNA (1 μL) was reverse-transcribed to cDNA and amplified by PCR. The target genes for amplification were iNOS, IL-1β, TNFα, IFNγ, IL-2, IL-4, IL-10, TGFβ2, IL-12p35, and IL-12p40. The primer sequences and amplification conditions are described in Table 1. Cycle numbers were determined empirically by sampling PCR amplification after every two cycles between 25 and 44, and selecting the approximate midpoint of linear amplification in relation to the isolation of a single band. The PCR products were electrophoresed on a 1.5% ethidium bromide-stained agarose gel and photographed under ultraviolet illumination using a Polaroid Type 667 positive/negative film. The intensity of the bands was scanned and converted into Macintosh format TIFF files (360 and 720 pixels with 8 bits of gray level) and exported to the public domain NIH image 1.55 for densitometric analysis. The expression levels of the target genes were calculated with the intensity of the β-actin bands.

### *Experimental Design*

On day 0, after intraperitoneal injection of 0.3 mL of 10% sodium pentobarbital, the anterior chamber of the right eyes of the BALB/c mice were inoculated with $5 \times 10^4$ PFU HSV-1 (KOS), as described previously. Mice were maintained for 7 days. On day 8 p.i., all mice were anesthetized and the contralateral eyes were examined clinically for evidence of retinitis. Only mice with clinical evidence of acute retinitis (70% of all mice inoculated with HSV-1) were used for further experiments.

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**Table 1.** Sequences of 5′ and 3′ Primers and Amplification Conditions

| Target | Primer Design | Expected Size of Product (base pairs) | Annealing Temperature (°C) | Cycles | Reference |
|---|---|---|---|---|---|
| $\beta$-actin | 5′ Primer 5′-ATG GAT GAC GAT ATC GCT-3′<br>3′ Primer 5′-ATG AGG TAG TCT GTC AGG T-3′ | 569 | 50 | 28 | Coligan et al[6] |
| iNOS | 5′ Primer 5′-ATG GCT TGC CCC TGG AAG TTT C-3′<br>3′ Primer 5′-CAA GAC TTG GAC TTG CAA GTG A-3′ | 326 | 60 | 36 | A kind gift from Department of 1st Internal Medicine, Kobe University School of Medicine |
| IL-1$\beta$ | 5′ Primer 5′-GCA ACT GTT CCT GAA CTC A-3′<br>3′ Primer 5′-GGC TGG ATT CCG GCA ACA-3′ | 382 | 55 | 30 | Coligan et al[6] |
| TNF $\alpha$ | 5′ Primer 5′-CAA AAG ATG GGG GGC TTC CAG AAC T-3′<br>3′ Primer 5′-AGT TAG CAA ATC GGC TGA CGG TGT G-3′ | 435 | 68 | 32 | M13049 |
| IFN $\gamma$ | 5′ Primer 5′-CGC TAC ACA CTG CAT CTT GG-3′<br>3′ Primer 5′-GGC TGG ATT CCG GCA ACA-3′ | 434 | 50 | 28 | Rabinovitch A et al[7] |
| IL-2 | 5′ Primer 5′-AAC AGC GCA CCC ACT TCA A-3′<br>3′ Primer 5′-TTG AGA TGA TGC TTT GAC A-3′ | 441 | 50 | 28 | Coligan et al[6] |
| IL-4 | 5′ Primer 5′-TAG TTG TCA TCC TGC TCT T-3′<br>3′ Primer 5′-CTA CGA GTA ATC CAT TTG C-3′ | 464 | 50 | 30 | Coligan et al[6] |
| IL-10 | 5′ Primer 5′-AGC TGG ACA ACA TAC TGA TAA CC-3′<br>3′ Primer 5′-CAC AGA TGT TCC GGT ACT TAC T-3′ | 301 | 50 | 30 | Rabinovitch A et al[7] |
| TGF $\beta$ 2 | 5′ Primer 5′-GTT GGG AAC GCG TTG CAT TT-3′<br>3′ Primer 5′-GCG CAT AAA CTG ATC CAT GT-3′ | 305 | 56 | 36 | Roelen BA et al[8] |
| IL-12p35 | 5′ Primer 5′-CTC AGT TTG GCC AGG GTC ATT C-3′<br>3′ Primer 5′-CCA AGG CAC AGG GTC ATC ATC-3′ | 308 | 60 | 37 | Sun B et al[9] |
| IL-12p40 | 5′ Primer 5′-AAA CAG TGA ACC TCA CCT GTG ACA C-3′<br>3′ Primer 5′-TTC ATC TGC AAG TTC TTG GGC G-3′ | 591 | 50 | 40 | M86671 |

iNOS: induced nitric oxide synthase, IL: interleukin, TNF: tumor necrosis factor, IFN: interferon, TGF: transforming growth factor.

Mice were sacrificed on days 0 (for control), 9, 11, 14, and 21 p.i. (n = 7 per time point). The uninoculated contralateral eyes were enucleated and extraocular tissues were dissected. The anterior and the posterior segments of the eyes were separated in Hank's balanced salt solution (GIBCO BRL). The posterior segment of each eye, containing the sclera, choroid, retina, and intraocular inflammatory cells, was used as samples in this study.

The experiment was repeated three times to confirm the results of each step of the study.

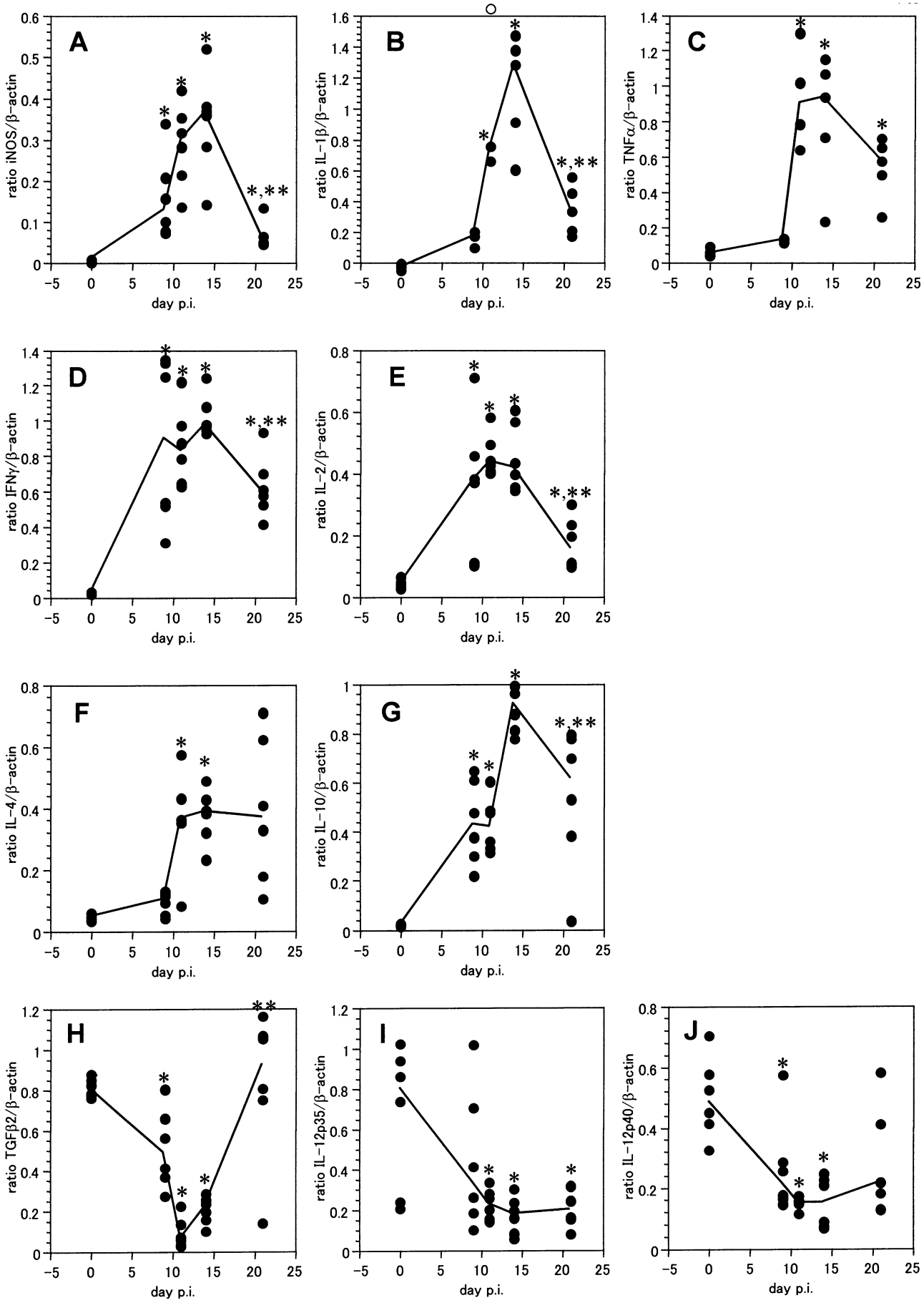
### *Analysis*

The Mann–Whitney *U*-test was used to determine significant differences in expression levels.

## Results

### *Expression of Cytokine mRNA and iNOS mRNA in the Contralateral Eyes*

The kinetics of mRNA expression of iNOS, IL-1β, TNFα, IFNγ, IL-2, IL-4, IL-10, TGFβ2, IL-12p35, and IL-12p40 in the posterior segments of the contralateral eyes is shown in the Figure 1. Expression of iNOS, IL-1β, and TNFα mRNAs was at very low levels in the normal eyes, and had demonstrated peaks at day 14 p.i. The cytokines mainly derived from lymphocytes, namely IFNγ, IL-2, IL-4, and IL-10, demonstrated similar results with peak expression at either day 11 or 14 p.i., coinciding with development of retinal necrosis. Expression levels for these cytokines decreased gradually by day 21 p.i. in the resolution phase. IL-12p35, IL-12p40, and TGFβ2 displayed a rather different mRNA expression kinetics. These cytokines were detected in the normal eyes with expression levels decreased during the ascending phase of the inflammatory process. On day 21 p.i., expression of TGFβ2 mRNA increased to the level of the normal eye at day 0, whereas IL-12p40 and IL-12p35 mRNA expression remained low. The comparison of mRNA expression was performed by statistical analysis, using the Mann–Whitney *U*-test ($P < .05$). For each cytokine and iNOS, the mRNA expression level at days 9, 11, 14, 21 p.i. was compared with that at day 0 in the normal eyes. To verify the significant increase in the resolution phase, mRNA expression at day 21 p.i. was compared with that of the peak. All the peak mRNA expression was


A
ratio iNOS/β-actin
day p.i.
B
ratio IL-1β/β-actin
day p.i.
C
ratio TNFα/β-actin
day p.i.
D
ratio IFNγ/β-actin
day p.i.
E
ratio IL-2/β-actin
day p.i.
F
ratio IL-4/β-actin
day p.i.
G
ratio IL-10/β-actin
day p.i.
H
ratio TGFβ2/β-actin
day p.i.
I
ratio IL-12p35/β-actin
day p.i.
J
ratio IL-12p40/β-actin
day p.i.

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statistically significant. mRNA expression of iNOS, IL-1β, TNFα, IFNγ, IL-2, and IL-10 decreased significantly at day 21 p.i.

## Discussion

Retinitis was experimentally induced in BALB/c mice by uniocular inoculation of HSV-1 (KOS).[1] The time course of the HSV-1 retinitis model is constant; the virus spreads to the contralateral retina by day 8 p.i., replicates massively at day 10 p.i. and is followed by fulminated intraocular inflammation.[3] This distinctive feature allows sampling of the inflamed eyes at a selected time period for detection and comparison of different factors. Most methods yield to technical limitations in assessing multiple factors, such as recruitment of inflammatory cells and cytokines with various bioactivities, in a small eyeball simultaneously. With the RT-PCR technique, amounts of mRNAs could be evaluated semiquantitatively and this technique is suitable for multifactorial analysis with a limited sample size.

CD4 T cells, an important factor in the inflammatory process, have been well studied in this HSV-1 retinitis model. It has been described that CD4 T cells contribute to the pathogenesis of this experimental model by modulating the HSV-1-induced retinitis to fulminant necrosis.[4] Immunohistochemical staining demonstrated that CD4 T cells infiltrate into the infected sensory retina at the onset of retinal necrosis.[5] Flow cytometric analysis,[5] however, revealed that the frequency of CD4 T cells maximized at day 21 p.i., which is the resolution phase. The discrepancy between the intraocular CD4 T cell frequency and the clinical activity of retinitis may mean that CD4 T cell frequency is not representative of the inflammatory activity. Expression of iNOS, which is well known for inducing overproduction of nitric oxide, causing various toxic effects through reactions with a superoxide, has been documented in viral infections, including HSV-1.[10] In the mouse influenza virus pneumonia model, the time profile of iNOS induction is parallel with that of pulmonary consolidation rather than with that of virus replication in the lung.[11] Our RT-PCR analysis of iNOS expression demonstrated upregulation of mRNA synthesis by day 14 p.i. with subsequent downregulation afterwards. This means that the kinetics of iNOS mRNA expression coincides with the activity of retinitis. The time course of iNOS synthesis, therefore, as a prototype of the kinetics of an inflammatory substance, is comparable with the time profiles of cytokine mRNA synthesis in the inflamed eye. As shown by our results, a similar pattern was observed in the kinetics of TNFα, IL-1β, IL-2, IL-4, IL-10, and IFNγ. However, mRNA expression of TGFβ2, IL12p35, and IL12p40 demonstrated a reversed time profile.

TNFα and IL-1β, the inflammatory cytokines derived mainly from mononuclear phagocytes, are known to be important links in acute inflammation and specific immune responses.[12] Because it has been previously described that natural-killer cells and macrophages are recruited intraocularly, preceding the infiltration of CD4 T cells in the sensory retina in the acute retinitis phase of this experimental model,[13] we predicted that the regulation of TNFα and IL-1β would occur in the early phase and that the peaks of mRNA production would come prior to those of cytokines from lymphocytes. Our data, however, showed the peak of mRNA synthesis at day 11 or 14 p.i., which implies that CD4 T cells boost the production of TNFα and IL-1β directly and indirectly.

Because anterior chamber-associated immune deviation (ACAID) was discovered in this experimental model of retinitis, a relationship has been presumed to exist between the disease in the contralateral eye and the suppression of delayed-type hypersensitivity (DTH) response specific to HSV-1 antigen.[14] In the previous study using CD4 T cell-depletion technique,[4] the crucial role of the T-cell subset in the development of retinal necrosis has been demonstrated. In explanation of the coexistence of active CD4 T cell function and the suppression of antigen-specific DTH response, it was predicted that the mRNA synthesis of IL-2 and IFNγ, which is associated with the TH1 response, would be reduced. Our data, however, demonstrates the mRNA expression not only of the TH2 cytokines, IL-4 and IL-10, but also of the TH1 cytokines, IL-2 and IFNγ, with kinetics which are parallel with the development of retinal necrosis. Intraocular production of both TH1 and TH2 cytokines, IFNγ and IL-10, has been supported by our recent data using enzyme-linked immunosorbant assay (ELISA).[15] The fact that IFNγ is produced intraocularly following the HSV-1 infection to the sen-

**Figure 1.** Time profile of mRNA expression of induced nitric oxide synthase (iNOS) (**A**), interleukin (IL)-1β (**B**), tumor necrosis factor (TNF)α (**C**), interferon (IFN)γ (**D**), IL-2 (**E**), IL-4 (**F**), IL-10 (**G**), transforming growth factor (TGF)β2 (**H**), IL-12p35 (**I**), and IL-12p40 (**J**) in murine herpes simplex virus (HSV)-1 retinitis eye. p.i: post inoculation, line: median of the intensity ratio to β-actin bands of individual samples at each day point, closed circles: the intensity ratio to β-actin bands of individual samples. *Comparison with normal level (day 0). $P < .05$. **Comparison with the peak level. $P < .05$.

sory retina does not conflict with the general idea that IFNγ is an important cytokine derived from CD4 T cells in immunity against viral infection. However, the fact is complex because the production of IFNγ, characteristic of TH1 response, was seen in mice with the anterior chamber-associated immune deviation (ACAID) induction demonstrated by suppression of the antigen-specific DTH response, which is considered to result from the TH1 response. It should be noted, however, that our data also show the remarkable synthesis of TH2 cytokine mRNAs, coinciding with that of TH1 cytokine mRNAs. IL-10, a TH2 cytokine, is a well-known inhibitor of IFNγ, and recent studies support the concept that TH2 lymphocytes secreting IL-10 are the immunosupressors.[16,17] It is presumed that ACAID induction specific for HSV-1 in this experimental model is established not by the suppression of TH1 response, generally dominant in viral infection, but by the induction of a strong TH2 response, which overwhelms the TH1 response.

TGFβ2 is an immunosuppressive factor normally produced in the eye that plays a key role in the immune privilege of the eye.[18] Expression of TGFβ2 mRNA demonstrated reverse kinetics in the inflammatory process of this experimental model. Because histopathological studies revealed that severe inflammation destroys the ocular parenchyma at day 14 p.i., downregulation of TGFβ2 mRNA production might be an event correlating with the dysfunction or the destruction of its intraocular sources during viral infection. Lower levels of mature TGFβ2 associated with uveitis have been also reported in the human eye.[19]

The kinetics of IL-12 mRNA showed a pattern similar to that of TGFβ2, suggesting that IL-12 is another cytokine derived from intraocular tissue. IL-12, a heterodimeric cytokine comprising p35 and p40 chains, is an immunoactivator, whose presence in the normal eye seems inappropriate considering the concept of immune privilege.[20,21] However, IL-12 is reported to be detectable in normal human aqueous humor using ELISA, both at the mRNA and the protein levels.[22] The reason for the intraocular production of IL-12 remains unknown, and it is unclear whether the IL-12 in the normal eye is in the active heterodimer form. A possible hypothesis may be that intraocular IL-12 exists in an inactive form or as a naturally occurring inhibitor, as IL-1,[23,24] antagonizing active IL-12, and contributing to the composition of the immune-privileged site.

At day 21 p.i., the resolution phase of retinitis, upregulation of TGFβ2 mRNA synthesis was observed, while expression of IL-12 mRNA remained low. These findings could be interpreted in two ways. One is that the restoration of the original intraocular source of TGFβ2 brings about the recovery of mRNA synthesis. The reason for failure of the recovery of IL-12 mRNA synthesis is that the sources of the two cytokines differ in the eye, and only the source of TGFβ2 can survive the severe destruction of ocular tissue. The other reason is that the original intraocular sources of both cytokines are totally damaged, leading to failure of cytokine synthesis thereafter. CD4 T cells, massively recruited into the eye by day 21 p.i., become the new source of TGFβ2 synthesis. Our data is compatible with the concept of regulatory T cells secreting high levels of TGFβ.[25] Although TGFβ derived from regulatory T cells is usually TGFβ1,[26,27] recent studies demonstrating large amounts of TGFβ2 secretion by T cells under distinctive condition[28–30] suggest that TGFβ2-producing intraocular CD4 T cells play an important role in inducing resolution of the inflammatory process by day 21 p.i.

In conclusion, we demonstrated the dynamic time profiles underlying the process of cytokine production in HSV-1 induced retinitis. Immunosuppressive cytokines such as IL-10 and TGFβ2 are also produced in transcriptional levels during the inflammatory process of this disease. The kinetics of TGFβ2 expression was out of phase with the other cytokines, suggesting that the resolution of inflammation may involve several factors expressed at different periods of the disease. Further studies aimed at identifying the intraocular sources of these cytokines are currently under way.

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