LABORATORY STUDY

Cytokine mRNA in BALB/c mouse corneas infected with herpes simplex virus

MARK HU, JAMES DUTT,
VICTOR ARRUNATEGUI-CORREA,
STEFANOS BALTATZIS,
C. STEPHEN FOSTER

Abstract

Purpose **To investigate cytokine mRNA expression and the influence of acyclovir and tetrandrine on that expression in the corneas of mice infected with herpes simplex virus type 1 (HSV-1).**

Methods **Male BALB/c mice were infected in the right cornea with HSV-1. The corneas were harvested from control normal mice and from untreated, acyclovir-treated and tetrandrine-treated mice 14 days after infection. The infected corneas of each group were divided into inflamed and uninflamed depending on clinical observation. After total mRNA extraction from the corneas, gene expression of interleukin 1β (IL-1β), IL-6, tumour necrosis factor α (TNF-α) and transforming growth factor (β (TGF-β) was analysed by reverse transcription polymerase chain reaction.**

Results **No mRNA expression of the cytokines was found in normal corneas. IL-1β and TNF-α mRNA was seen in inflamed corneas, while mRNA expression of IL-6 and relatively weaker TGF-β mRNA expression were found both in inflamed corneas and in infected but uninflamed corneas treated with acyclovir. TNF-α mRNA was present in the uninflamed corneas of tetrandrine-treated mice. No influence of either agent was found on TGF-β gene expression.**

Conclusions **The results suggest that local IL-1β and TNF-α gene expression is required for corneal inflammation, whereas IL-6 and TGF-β may exert antiviral and inflammation regulatory activities in HSV corneal infection.**

Key words Cytokine gene expression, Herpes simplex virus, Keratitis, Mouse, Tetrandrine

Herpes simplex virus (HSV)-induced keratitis (HSK) occurs largely secondary to an immunologically driven inflammatory response to the virus.[1–5] Although much is now known about systemic immunity and cytokine response to viral infection, little is known about the regional cytokine gene expression in the cornea following corneal inoculation with HSV. The mechanisms of defence against HSV infection almost certainly involve not only the development of a systemic immune response to HSV but also the local secretion of cytokines with antiviral activity. Interleukin–1 (IL-1), tumour necrosis factor α (TNF-α) and IL-6 are mainly produced by cells of monocyte lineage and are considered to be major mediators of inflammation.[6,7] It is suggested that they may display a similar but not identical spectrum of activities including differentiation and growth-regulating activities, inflammatory and anti-inflammatory, haematopoietic, anti-tumoural and antiviral activities. However, their roles in inflammation, particularly in HSV-infected corneas, remain to be elucidated.

IL-1 is widely regarded as a primary mediator of specific and non-specific inflammation, and transforming growth factor β (TGF-β) is a well-known potent down-regulator of inflammatory reactions, inhibiting B- and T-cell proliferation, cytokine production and many cytokine-mediated immune responses.[8] The importance of TGF-β in controlling inflammatory responses is highlighted by the finding that mice with a targeted disruption of the TGF-β gene suffer from an acute wastage syndrome characterised by inflammatory cell infiltration and tissue necrosis.[9] The roles of IL-6 and TNF-α are not yet fully understood. Reports increasingly indicate that IL-6 and TNF-α can be anti-inflammatory and immunosuppressive.[10–14]

Some inbred strains of mice can tolerate HSV infection, with no clinically evident inflammation of the infected corneas, while others develop necrotising stromal keratitis after HSV corneal inoculation. Indeed this difference in HSK susceptibility resides in the fine specificity of gene loci at or near the Igh-1 locus on chromosome 12.[15] A novel anti-inflammatory agent, tetrandrine, isolated from an ancient Chinese herbal remedy, is a potent inhibitor of the destructive inflammation typical of HSK in genetically susceptible mice;[16] and acyclovir, of course, is also therapeutically beneficial for murine HSK. The objectives of the present study were to evaluate and compare the difference in local cytokine gene expression between inflamed and non-inflamed corneas,

M. Hu
J. Dutt
V. Arrunategui-Correa
S. Baltatzis
C.S. Foster
Rhoads Molecular and Hilles Immunology Laboratories
Department of Ophthalmology
Massachusetts Eye & Ear Infirmary
Harvard Medical School
243 Charles Street
Boston, MA 02114, USA

Dr C. Stephen Foster, MD, FACS ✉
Immunology Service
Department of Ophthalmology
Massachusetts Eye & Ear Infirmary
Harvard Medical School
243 Charles Street
Boston, MA 02114, USA

This study was supported in part by an unrestricted grant from Research to Prevent Blindness, Inc. (New York, NY)

Received: 8 July 1998
Accepted in revised form: 29 January 1999

Table 1. *Oligonucleotide primers*

	Oligonucleotide sequence	PCR product (bp)
GAPDH		
Sense:	5′–CCA TGG AGA AGG CTG GG	
Anti-sense:	5′–CAA AGT TGT CAT GGA TGA CC	195
IL-1β		
Sense:	5′–ATG GCA ACT GTT CCT GAA CTC	
Anti-sense:	5′–CAG GAC AGG TAT AGA T TC TTT C	563
IL-6		
Sense:	5′–TTC CTC TCT GCA AGA GAC T	
Anti-sense:	5′–TGT ATC TCT CTG AAG GAC T	432
IL-α		
Sense:	5′–TTC TGT CTA CTG AAC TTC GGG GTF ATC GGT CC	
Anti-sense:	5′–GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG	354
IL-β		
Sense:	5′–TGG ACC GCA ACA ACG CCA TCT ATG AGA AAA CC	
Anti-sense:	5′–TGG AGC TGA AGC AAT AGT TGG TAT CCA GGG CT	525

and to examine the influence of tetrandrine and acyclovir on the expression of these cytokines in HSV-infected corneas.

Materials and methods

Virus

The HSV-1 (KOS strain) stock was obtained from Dr Priscilla Schaffer (Harvard Medical School, Boston), grown in our laboratory, and passed twice in Vero cell monolayers (American Type Culture Collection, ATCC, CCL 81, Rockville, MD), as previously described.[1,16] The same virus suspended in Eagle's Minimum Essential Medium (MEM) was used in all experiments.

Pharmacological agents

Tetrandrine (Aldrich Chemical Company, Milwaukee, WI), was used after conversion to the hydrochloride form, soluble in water. Acyclovir (Burroughs Wellcome, Research Triangle Park, NC) was freshly dissolved in distilled water as a 1.5% solution before injection.

Animals

Male BALB/C mice 6–8 weeks of age were obtained from The Jackson Laboratories (Bar Harbour, ME) and housed in microisolators mounted in ventilated animal racks in our facility. All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal inoculation

Corneal infection of HSV-1 and treatment were performed as previously described.[1,16] Briefly, mice were anaesthetised with 2 mg of intraperitoneal ketamine hydrochloride (Ketalar, Parke-Davis, Morris Plains, NJ) and 400 mg of xylazine (Rompun, Mobay, Shawnee, KD). The right cornea of each mouse was scratched eight times in a criss-cross pattern with a 25-gauge needle, and 5 μl of HSV-1 suspension containing 10^5 plaque-forming units was instilled in the cul-de-sac as previously described.[1,16] Twenty mice infected with HSV-1 were injected intraperitoneally (i.p.) with distilled water and served as controls. The second group of 20 infected mice were injected (i.p.) with acyclovir 60 mg/kg twice daily from day 0, and the third group of 20 infected mice were injected with tetrandrine 15 mg/kg twice daily from day 7 after infection. Eyes of the mice were observed with an operating microscope every other day. At day 14 after HSV inoculation, the corneas ($n = 8$) of each group were divided into inflamed and non-inflamed groups based on clinical observation, and harvested separately for cytokine mRNA analysis.

Cytokine mRNA expression

mRNA extraction, cDNA synthesis and polymerase chain reaction (PCR) amplification were performed as previously described.[17] Briefly, the corneas harvested on day 14 after infection were pooled by group, and put into RNAzol B solution (TEL-TEST, Friendswood, TX) that was maintained at 4 °C, followed by homogenisation in the solution, extraction with phenol and chloroform, and precipitation with isopropanol. Total RNA (2.0 μg) was used for synthesis of cDNA. Reverse transcription mix consisting of: 4 μl 5× transcriptase buffer (Boehringer Mannheim, Indianapolis, IN), 2 μl dNTPs (5 mM; Boehringer), 1 μl oligo dt (18 mer), 0.5 μl RNAse inhibitor (40 U/μl; Promega) and 1.0 μl of AMV reverse transcriptase (24 U/μl; Boehringer) was added. The reverse transcription was allowed to proceed at 37 °C for 60 min. cDNA samples were diluted 1/5 in distilled water and 20 μl of this dilution was subjected to PCR amplification.

PCR amplification

The sense and anti-sense sequences of the oligonucleotide primers used, and the size of amplified PCR products (bp) for each pair of primers, are shown in Table 1; these sequences were chosen from published sequences in GenBank and synthesised on a Gene

Table 2. *Cytokine mRNA expression in murine corneas*

	n	IL-1β	TNF-α	IL-6	TGF-β
Normal controls	8	–	–	–	–
Infected					
Distilled water					
Uninflamed	8	–	–	++++	++
Inflamed	8	+++[a]	+++	++++	++
Acyclovir					
Uninflamed	8	–	–	+[b]	++
Tetrandrine					
Uninflamed	8	–	++	–	++
Inflamed	8	++++	+++	++++	++

[a] + to ++++ is based on the intensity of the polymerase chain reaction product gel band.
[b] + uninflamed indicates no obvious clinical inflammation.

Assembler DNA synthesiser (Parmacia LKB, Piscataway, NJ). PCR was performed with AmpliTaq DNA polymerase (Boehringer, Indianapolis, IN) at 2 units/reaction and 10 μl of cDNA. The total reaction volume was 50 μl in each tube. The reactions were conducted in a Perkin Elmer Thermal Cycler Model 9600 (Perkin, Elmer Cetus, Norwalk, CT) with the following profile: 35 cycles of denaturation for 45 s at 94 °C, 45 s annealing at 60 °C, and 2 min elongation at 72 °C.

Semi-quantitative comparisons of mRNA content

β-Actin mRNA was amplified from each sample as quality control cDNA and also to adjust for differences in the amount of cellular RNA isolated during the RNA extraction; the β-actin 'housekeeping' gene product was used to normalise the amount of RNA for every sample. Total RNA for cytokine testing was first semi-quantitated by PCR of β-actin RNA and normalised to these amounts. After co-amplification of each cytokine's primers with β-actin primers, the density of the bands from Southern blot films was analysed by scanning densitometry using a personal densitometer (Molecular Dynamics, Sunnydale, CA). The results of gene expression were determined by direct comparison of the ratios between both PCR products (cytokine and internal control). Using this method, we have been able to semi-quantify mRNA levels and demonstrate kinetic behaviour in transcription of each sample at determined time points.[18] We express the results (Table 2) on a zero to 4+ semi-quantitative scale.

Results

Clinical observation

HSK had developed in 70% of the untreated mice by 14 days after inoculation. The onset of keratitis was at days 8 to 10 after corneal infection, which was in accordance with our previous studies.[1,2,16] No mice receiving acyclovir from day 0 developed HSK; the incidence of HSK was 10% in mice treated with tetrandrine from day 7. This result was in accordance with our previous observations.[16]

Cytokine mRNA expression in corneas

Housekeeping gene glycerine-3′-phosphate dehydrogenase (GAPDH) mRNA was detected in every group of the corneas as expected. mRNA expression of all the cytokines studied was not found in normal

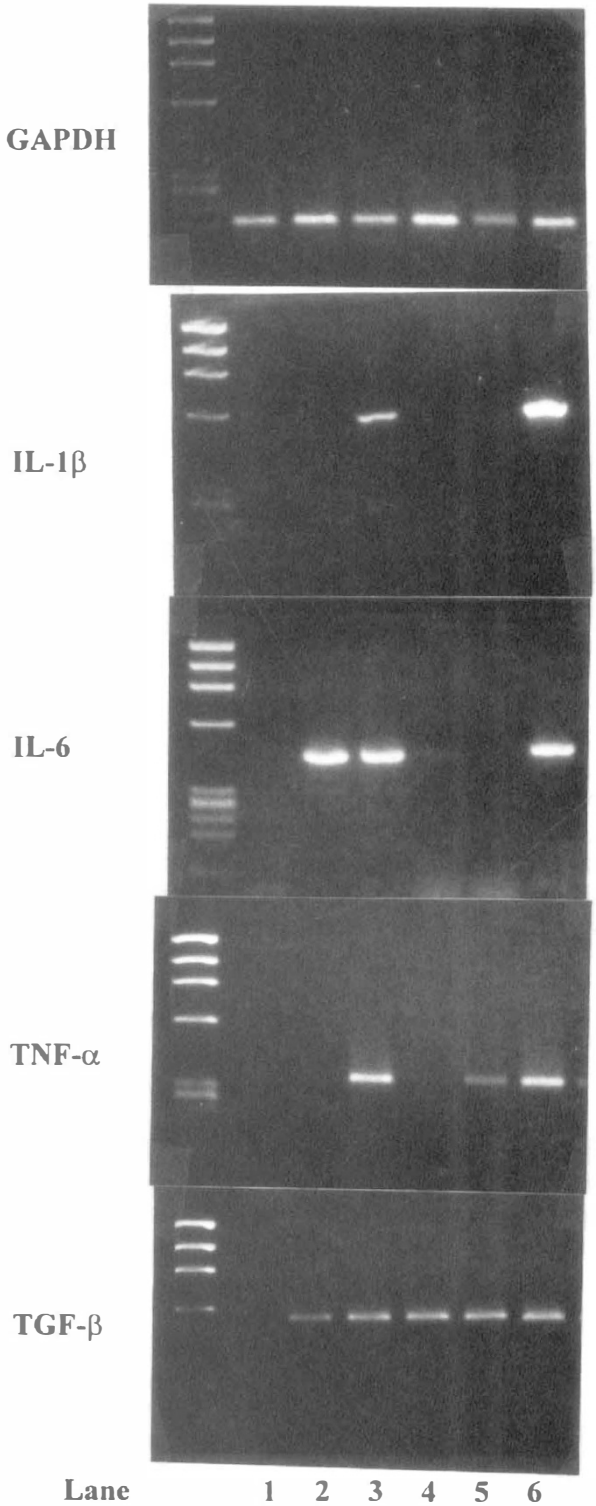

Fig. 1. *Agarose gel electrophoresis patterns of PCR products of cytokine mRNA in the corneas. Lane 1, normal corneas; lane 2, infected corneas with no inflammation; lane 3, infected corneas with inflammation (HSK); lane 4, non-inflamed corneas treated with acyclovir from day 0; lanes 5 and 6, non-inflamed corneas and inflamed corneas treated with tetrandrine from day 7. X174 DNA/Hae markers (0.07-1.35 kb) were used.*

corneas. In the control group treated with distilled water, mRNA of IL-1β, TNF-α, IL-6 and TGF-β was expressed in the infected and inflamed corneas; intense IL-6 mRNA and relatively weaker TGF-β mRNA was found as well in the infected but non-inflamed corneas. This result suggests that expression of IL-6 and TGF-β mRNA in response to HSV infection is not, but that of IL-1β is, coincident with clinically obvious inflammatory destruction of the cornea. TNF-α mRNA expression was weakly detected in non-inflamed corneas of tetrandrine-treated mice. IL-6 was found in the non-inflamed corneas of acyclovir-treated mice. Consistent TGF-β mRNA expression was detected in all the infected corneas with and without inflammation of either treated or untreated mice. There was no significant difference between the groups of infected mice, and the expression of this cytokine in the infected cornea was not influenced by either tetrandrine or acyclovir (Fig. 1, Table 2).

Discussion

It was reported that mRNA of T cell cytokines including IL-2, IL-4, IL-5, IL-10 and interferon gamma in mouse corneas was expressed and IL-10 may be involved in HSK resolution during the course of immunopathological herpetic stromal keratitis.[19] The present study demonstrates significant local gene expression of IL-1β, IL-6, TNF-α and TGF-β in response to HSV-1 infection in mouse cornea. Although cytokine protein production was not examined in this study, it is likely that the cytokine gene induction resulted in the actual release of cytokine proteins. The release of IL-1 and IL-6 together with TNF-α and TGF-β may contribute both to the antiviral response to HSV-1 infection and to the corneal damage. The gene expression pattern of the cytokines suggests that they play distinct roles individually in the pathogenic process of HSK in mice. IL-1 and TNF-α are mainly produced by mononuclear leucocytes and are considered to be major mediators of the inflammatory processes.[6,7,20] IL-1 and TNF-α are sometimes referred to as 'master' cytokines because of their ability to govern the expression of several other cytokines, including IL-6. Since IL-1β mRNA expression was only associated with, and was especially prominent in, the inflamed corneas, it appears that this cytokine is essentially required for induction of the corneal inflammation.

IL-6 acts as a critical anti-inflammatory regulator in both local and systemic acute inflammatory responses by controlling the level of pro-inflammatory, but not anti-inflammatory responses by controlling the level of pro-inflammatory, but not anti-inflammatory cytokines.[12] IL-6 also protects mice against experimental bacterial infection[13] and against enterotoxin-induced toxic shock.[14] A recent report indicates that IL-6 is not sufficient for inducing uveitis in mice,[21] implying its possible regulatory role in the eye. Our results suggest that IL-6 and TGF-β may function as anti-inflammatory mediators for protection against the virus-induced inflammation and for modulation of other pro-inflammatory cytokines in the cornea. The observation that an intense signal of IL-6 mRNA expression was obtained not only in the clinically inflamed corneas but also in the non-inflamed but infected corneas suggests that this cytokine can be produced by keratocytes and may be a beneficial regulator that suppresses inflammation and protects the cornea rather than an aggressive stimulator in the inflammatory process. This possibility was reinforced by the finding that expression of IL-6 was present in the non-inflamed corneas of acyclovir-treated mice. The results agree with the viewpoint that IL-6 functions as a pleiotropic immunoregulatory lymphokine and as an exocrine hormone in inflammation as indicated above.[21–24] In addition, the observation that no or significantly reduced IL-6 mRNA expression in the uninflamed corneas of tetrandrine- or acyclovir-treated group was detected suggests that these two compounds may modulate local IL-6 mRNA expression.

TNF has been found to play a key role in orchestrating the complex events involved in inflammation and immunity. Most attention has been focused on the pro-inflammatory actions of TNF-α. But TNF-α has also been reported to play a crucial role in the development of anterior-chamber-associated immune deviation (ACAID),[25] suggesting its potential in the down-regulation of infection in the eye. A recent study with TNF-α-deficient mice indeed indicates that this cytokine has an essential homeostatic role in limiting the extent and duration of an inflammatory process, i.e. an anti-inflammatory function.[11] Expression of TNF-α in the non-inflamed corneas treated with tetrandrine suggest that this cytokine, as well as IL-6, may play a role, or at least is not sufficient for induction of corneal inflammation. This dual function potential of TNF – pro-inflammatory in the initial phase of infection and inflammation and anti-inflammatory after the infectious or toxic agent has been localised and controlled – has been noted previously.[11] Such a dual function, presumably dose-dependent as well as phase-dependent, may occur in HSK.

We believe the suppressed expression of IL-1β, IL-6 and TNF-α by acyclovir resulted from its effective inhibition of viral replication. Tetrandrine reportedly exhibits potent inhibitory effects of IL-1 and TNF-α release from monocytes.[26,27] Despite the fact that we have shown consistent inhibition of HSV-mediated destructive keratitis by tetrandrine therapy, in this study we are unable to identify the effect of tetrandrine on IL-1β and TNF-α gene expression, since there was no expression of either cytokine in the uninflamed corneas of the untreated mice. However, our finding demonstrated clearly that tetrandrine inhibited mRNA expression of IL-6 in the cornea. It is interesting to note that TGF-β mRNA was consistently expressed in response to the infection and was not changed in the inflamed and uninflamed corneas, and even not influenced by either of the compounds used in this study. The detailed mechanisms of tetrandrine inhibition of destructive keratopathy in murine HSK remain to be elucidated.

Investigations with cytokine-gene-deficient mice are under way to further investigate the role of these and other cytokines in the pathogenesis of HSK, and the mechanism of action of tetrandrine.

We fully recognise that this is a preliminary study of a limited cytokine panel with semi-quantitative PCR. We are currently in the process of examining our expanded panel of cytokines in the model with internally nested controls in a quantitative PCR technique. However, we think that sharing the preliminary results of the effects of an interesting, novel immunomodulator (tetrandrine) with the vision research community is of substantial interest.

References

1. Foster CS. Herpes simplex virus-induced destructive corneal disease. Eye 1989;3:194–203.
2. Jayaraman S, Heiligenhaus A, Rodriguez A, Soukiasian S, Dorf ME, Foster CS. Exacerbation of murine herpes simplex virus-mediated stromal keratitis by the TH2 type T cells. J Immunol 1993;151:5777–89.
3. Niemialtowski MG, Rouse BT. Phenotypic and functional studies on ocular T cells during herpetic infections of the eye. J Immunol 1992;148:1864–70.
4. Streilein JW, Dana MR, Ksander BR. Immunity causing blindness: five different paths to herpes stromal keratitis. Immunol Today 1997;18:443–9.
5. Hendricks RL, Tumpey TM. Contribution of virus and immune factors to herpes simplex virus type 1-induced corneal pathology. Invest Ophthalmol Vis Sci 1990;31:1929–39.
6. Sprecher E, Becker Y. Detection of IL-1β, TNF-α and IL-6 gene transcription by the polymerase chain reaction in keratinocytes, Langerhans' cells and peritoneal exudate cells during infection with herpes simplex virus-1. Arch Virol 1992;126:253–69.
7. Higuchi M, Aggarwal BB. Differential roles of two types of the TNF receptor in TNF-induced cytotoxicity, DNA fragmentation, and differentiation. J Immunol 1994;152:4017–25.
8. Barnum SR, Jones JL. Transforming growth factor-β1 inhibits inflammatory cytokine-induced C3 gene expression in astrocytes. J Immunol 1994;152:765–73.
9. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, *et al*. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature 1992;22:693–9.
10. Tilg H, Dinarello CA, Mier JM. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. Immunol Today 1997;18:428–32.
11. Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, *et al*. Characterisation of tumour necrosis factor-deficient mice. Proc Natl Acad Sci USA 1997;94:8093–8.
12. Xing Z, Gualdie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest 1998;101:311–20.
13. Liu Z, Simpson RJ, Cheers C. Recombinant interleukin-6 protects mice against experimental bacterial infection. Infect Immun 1992;60:4402–6.
14. Barton BE, Shortall J, Jockson JV. Interleukin 6 and 11 protect mice from mortality in a staphyloccal enterotoxin-induced toxic shock model. Infect Immun 1996;64:714–8.
15. Foster CS, Opremcak EM, Rice B, Wells P, Chung H, Thompson P, *et al*. Clinical, pathologic, and immunopathologic characteristics of experimental murine herpes simplex virus stromal keratitis and uveitis is controlled by gene products from the Igh-1 locus on chromosome 12. Trans Am Ophthalmol Soc 1987;85:293–311.
16. Hu S, Dutt J, Zhao T, Foster CS. Tetrandrine potently inhibits herpes simplex virus type-1-induced keratitis in BALB/c mice. Ocul Immunol Imflamm 1997;5:173–80.
17. Hu S, Merayo-Lloves J, Zhao T, Foster CS. Potent inhibitory effect tetrandrine on experimental allergic conjunctivitis in mice. J Ocul Pharmacol Ther 1997;13:435–44.
18. Power WJ, Kaufman AH, Merayo-Lloves J, Arrunategui-Correa V, Foster CS. Expression of collagens I, III, IV and V mRNA in excimer wounded rat cornea: analysis by semi-quantitative PCR. Curr Eye Res 1995;14:879–86.
19. Babu JS, Kanangat S, Rouse BT. T cell cytokine mRNA expression during the course of the immunopathologic ocular disease herpetic stromal keratitis. J Immunol 1995;154:4822–9.
20. Wong CW, Seow WK, Zeng TS, Halliday WJ, Thong YH. Comparative immunopharmacology and toxicology of the bisbenzylisquinoline alkaloids tetrandrine and berbamine. Int J Immunopharmacol 1991;13:579–85.
21. Rosenbaum JT, Kievit P, Han YB, Park JM, Planck SR. Interleukin-6 does not mediate endotoxin-induced uveitis in mice: studies in gene deletion animals. Invest Ophthalmol Vis Sci 1998;39:64–9.
22. Planck SR, Huang XN, Robertson JE, Rosenbaum JT. Cytokine mRNA levels in rat ocular tissues after systemic endotoxin treatment. Invest Ophthalmol Vis Sci 1994;35:924–30.
23. Gauldie J, Northemann W, Fey GH. IL-6 functions as an exocrine hormone in inflammation: hepatocytes undergoing acute phase responses require exogenous IL-6. J Immunol 1990;144:3804–8.
24. Podor TJ, Hirsh J, Gelehrter TD, Zeheb R, Torry D, Guigoz Y, *et al*. Type 1 plasminogen activator inhibitor is not an acute phase reactant in rats: lack of IL-6 and hepatocyte-dependent synthesis. J Immunol 1993;150:225–35.
25. Ferguson TA, Herndon JM, Dube P. The immune response and the eye: a role for TNF-α in anterior chamber-associated immune deviation. Invest Ophthalmol Vis Sci 1994;35:2643–51.
26. Seow WK, Ferrante A, Li SY, Thong YH. Suppression of human monocyte interleukin-1 production by the plant alkaloid tetrandrine. Clin Exp Immunol 1989;75:47–51.
27. Ferrante A, Seow WK, Rowan-Kelly B, Thong YH. Tetrandrine, a plant alkaloid, inhibits the production of tumour necrosis factor-α (cachectin) by human monocytes. Clin Exp Immunol 1990;80:232–5.