

Characterization of *Toxoplasma gondii*-Specific T Cells Recovered from Vitreous Fluid of Patients with Ocular Toxoplasmosis

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PURPOSE. The mechanisms involved in reactivations of latent ocular *Toxoplasma gondii* (Tg) infections in immunocompetent patients are poorly understood. In view of the possible role of T cells in the immunopathogenesis of the disease, ocular infiltrating T cells obtained from patients with recurrent ocular toxoplasmosis were characterized phenotypically and functionally.

METHODS. Ocular infiltrating T cells were recovered from vitreous fluid (VF) samples of 10 patients with active recurrent ocular toxoplasmosis. Two patients with uveitis of other origins were included as control subjects. T-cell lines (TCLs) were generated by mitogenic stimulation and tested for reactivity to Tg and human retinal protein extracts. The TCLs of three patients were cloned by limiting dilution. Tg-reactive T-cell clones (TCCs) were characterized with respect to their phenotype, T-cell receptor variable (TCR V)- β gene usage, HLA restriction, and cytokine secretion profile.

RESULTS. Reactivity to Tg could be detected only in the TCLs of patients with ocular toxoplasmosis. None of the TCLs showed reactivity to human retinal antigens. All tested intraocular Tg-specific TCCs ($n = 23$) were CD3⁺CD4⁺ and displayed differential TCR V β usage. Twenty-one TCCs were HLA-DR restricted and two TCCs were restricted by HLA-DP. The majority of the intraocular Tg-specific TCCs showed a bias toward a T-helper (Th)0-Th2 cytokine profile.

CONCLUSIONS. The data indicate that T cells specific for the triggering microorganism infiltrate the eye of patients with recurrent ocular toxoplasmosis. The functional characteristics of the VF-derived Tg-specific T cells and their presence at the site of inflammation suggest their involvement in the local inflammatory response of ocular toxoplasmosis. (*Invest Ophthalmol Vis Sci.* 2001;42:3228–3232)

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Toxoplasmosis is caused by the obligate intracellular protozoan *Toxoplasma gondii* (Tg). It is estimated that at least 500 million people worldwide are infected with Tg.¹ Human infection may occur either by the congenital or acquired route. Congenital toxoplasmosis is caused by transplacental transmission of Tg tachyzoites, whereas acquired infection results from ingestion of food, soil, or water contaminated with Tg cysts or oocysts.² Infection of an immunocompetent host normally induces a strong type 1 T-cell (Th1)-mediated immune response. This response is characterized by high levels of interferon (IFN)- γ , involved in killing of intracellular residing parasites. This rapidly controls the dividing tachyzoite stage of the parasite.³ The parasite may persist for life in the host by sequestration within tissue cysts containing the quiescent bradyzoite stage. The high frequency of Tg cysts in the eye and the brain compared with extraneural tissues may be related to the immune privileged state of these tissues in which inflammatory responses are actively suppressed.⁴

Ocular infection with Tg is a major cause of visual impairment throughout the world.⁵ Most cases of ocular toxoplasmosis are believed to result from reactivation of latent congenital or acquired infections.⁶ T cells have been suggested to play a pivotal role in these recurrences.^{3,7} Flow cytometric analyses have shown the predominance of T cells among the inflammatory cells located in the aqueous humor in patients with active disease,⁸ and the percentages of activated T cells in the eye are higher than in the peripheral blood.^{9,10} Thus far, the antigen specificity and functional properties of these ocular infiltrating T cells have not been analyzed.

To determine the role of T cells in the immunopathology of recurrent ocular toxoplasmosis, a study was designed to isolate and characterize the ocular infiltrating T cells recovered from the vitreous fluid (VF) of 10 patients with active disease. As a control, two patients with different origins of uveitis were included. We have tested whether the VF-derived T-cell lines (TCLs) and resultant T-cell clones (TCCs) were specific for Tg antigens or human retinal antigens. The TCC were characterized with respect to their phenotype, T-cell receptor variable (TCR V)- β gene usage, HLA-restriction of antigen recognition, and cytokine secretion profile.

MATERIALS AND METHODS

Patients

Ten immunocompetent patients included in this study had unilateral presumed ocular toxoplasmosis and underwent a surgical pars plana vitrectomy for therapeutic or diagnostic reasons during an episode of active retinitis lasting for a minimum of 1 week at the time of vitrectomy. The characteristics, diagnosis, reasons for surgical intervention, and preoperative treatment of the patients studied are listed in Table 1. The diagnosis of ocular toxoplasmosis was based on the typical aspect of the retinal lesions: full-thickness necrotizing retinitis, adjacent to a chorioretinal scar, with overlying vitreous cellular reaction. In some cases, a diagnostic vitrectomy was performed by the surgeon to ex-

TABLE 1. Characteristics of Patients with Ocular Toxoplasmosis and Control Subjects Included in the Present Study

Patient	Sex	Age (y)	Disease Activity*	Reason for Vitrectomy	Preoperative Treatment	G-WC†	PCR Tg‡	Clinical Diagnosis
1	M	32	1	Macular pucker	None	39	–	Toxoplasmosis
2	F	62	2	Diagnostic	Subconjunctival betamethasone, topical prednisolone	6	+	Toxoplasmosis
3	F	75	2	Diagnostic	Topical prednisolone	11	+	Toxoplasmosis
4	F	29	4	Diagnostic	Topical prednisolone	15	ND	Toxoplasmosis
5	M	26	2	Diagnostic	Systemic prednisone, pyrimethamine, sulphadiazine, topical prednisolone	40	–	Toxoplasmosis
6	F	66	3	Diagnostic	Subconjunctival betamethasone, topical dexamethasone	22	+	Toxoplasmosis
7	M	22	2	Retinal detachment	Topical dexamethasone	12	–	Toxoplasmosis
8	F	29	2	Retinal detachment	Topical prednisolone	7	–	Toxoplasmosis
9	F	37	3	Diagnostic	Topical prednisolone	111	–	Toxoplasmosis
10	M	20	2	Retinal detachment	Systemic prednisone, pyrimethamine, sulphadiazine, topical prednisolone	27	ND	Toxoplasmosis
11	F	59	3	Diagnostic	Topical dexamethasone	<1	–	Panuveitis
12	F	52	3	Diagnostic	Systemic prednisone	<1	ND	Pars planitis

* Disease activity was assessed by grading the severity of vitreous activity, as measured by the amount of vitreous cells and haze, using the criteria of Nussenblatt et al.³³

† Goldmann-Witmer coefficient to *T. gondii*.

‡ + or –, positive or negative *T. gondii*-specific PCR product detected. ND, not done.

clude other causes of necrotizing retinitis (patients 2, 3, and 5) or because the density of the vitreous haze precluded visibility of the fundus (patients 4, 6, and 9). In all patients, the clinical diagnosis was confirmed by paired serologic examination of VF and serum samples,¹¹ which showed evidence of intraocular synthesis of antibodies against Tg (Goldmann-Witmer coefficient = antibody titer VF/serum × total IgG serum/VF ≥ 3; Table 1). The presence of Tg DNA in the VF samples was determined by PCR, as described previously (Table 1).¹¹ The present study was performed according to the Declaration of Helsinki, and informed consent was obtained from all patients. Two patients with uveitis of different origins (i.e., panuveitis and pars planitis), who were undergoing a vitrectomy, were included in the study as negative control subjects for proliferation of intraocular T cells in response to Tg antigen (Table 1; patients 11 and 12).

Collection of VF and Peripheral Blood

Undiluted VF was washed twice with RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% pooled heat-inactivated normal human serum (NHS; Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands) and antibiotics. This medium is further referred to as culture medium. Peripheral blood was collected into heparinized tubes, and peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation over a single-density gradient (Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and stored in liquid nitrogen for further use.

Generation of TCLs and TCCs

The TCLs were obtained by nonspecific stimulation of VF-derived cells with 10 μg/ml phytohemagglutinin (PHA; Gibco), in the presence of a feeder mixture of γ-irradiated allogeneic PBMCs (3000 rad; 10⁵/well), in culture medium supplemented with 20 U/ml recombinant interleukin (rIL)-2 (Gibco). The T-cell cultures were incubated in 96-well, round-bottomed tissue culture plates (Costar, Cambridge, MA) for 2 weeks at 37°C in a humidified atmosphere containing 5% CO₂.

The TCCs were generated from the VF-derived TCLs by limiting dilution in microwell plates (Terasaki-Microwell; Nunc, Roskilde, Denmark) in the presence of irradiated allogeneic PBMCs, PHA, and rIL-2 as described by Hermann et al.¹² After 14 days, expanding clones were

transferred to 96-well plates in culture medium supplemented with 20 U/ml rIL-2. The TCCs were restimulated every 10 to 14 days with 1 μg/ml PHA in the presence of a feeder mixture of irradiated allogeneic PBMCs, as described by Verjans et al.¹³ Only in patients 1, 4, and 8 were TCCs generated in sufficient numbers to make subsequent testing possible.

Tg and Human Retinal Antigen Extracts

A crude Tg antigen preparation was a gift of Frans van Knapen (Department of Parasitology, National Institute of Public Health, Bilthoven, The Netherlands) and was obtained as previously described by Hughes et al.¹⁴ Briefly, tachyzoites of the RH strain of Tg were harvested from the peritoneal exudates of Tg-infected mice, washed with saline, and disrupted by repeated cycles of freeze thawing. Human retinal extract was obtained from surplus material of donor eyes, as described previously.¹⁵

T-Cell Proliferation Assay

Ten to 14 days after the last stimulation, T cells were washed three times in RPMI-1640 plus 5% heat-inactivated fetal calf serum (FCS; Gibco) to remove all rIL-2. The antigen specificity of the TCLs and TCCs was assayed in triplicate by culturing the T cells (2 × 10⁴/well) in culture medium in 96-well, round-bottomed culture plates in the presence of specific antigens and γ-irradiated autologous PBMCs (3000 rad; 10⁵/well) as a source of antigen-presenting cells. The cells were cultured for 3 days at 37°C in a CO₂-incubator and labeled with [³H]-thymidine (1 μCi/well; Amersham International, Amersham, UK) during the last 18 hours of incubation. The Tg antigen and human retinal protein extracts were used at final concentrations of 10 and 100 μg/ml respectively, which had been shown to yield maximal T-cell proliferation in preliminary experiments (data not shown). Subclasses of HLA class II restriction determinants were characterized by blocking the Tg-specific T-cell proliferation with appropriate dilutions of the following monoclonal antibodies (mAbs): anti-HLA DR (B.8.11.2; a gift of Frits Koning, Department of Immunohematology, University of Leiden, The Netherlands), anti-HLA-DQ (SPV L3-8; a gift of Hergen Spits, The Netherlands Cancer Institute, Amsterdam, The Netherlands) and anti-HLA-DP (B21/7; Becton Dickinson, Mountain View, CA) in a standard proliferation assay. The stimulation index (SI) was calculated as the ratio of antigen-stimulated proliferation to the background pro-

liferation—T cells incubated with PBMCs without antigen. An SI of more than three was considered positive. The SD in all assays was less than 15% of the mean value.

T-Cell Phenotype and TCR V β Gene Usage

T cells were labeled with fluorescein-conjugated mAb against CD4 and CD8 or phycoerythrin-conjugated mAb directed toward CD3 (Dako, Glostrup, Denmark). The labeled cells were analyzed by flow cytometry (FACScan; Becton Dickinson).

The TCR V β gene usage of the TCCs was determined by RT-PCR as described previously.¹⁶ Briefly, total RNA was isolated from 10^5 to 10^6 T cells by use of extraction agent (RNAzol; Campro Scientific, Elst, The Netherlands) and cDNA generated by using reverse transcriptase and oligo-dT (Gibco). For the PCR amplification on cDNA, 23 TCR V β -specific 5' sense primers in combination with a 3' antisense C β primer were used.¹⁶ PCR products were fractionated according to size by electrophoresis on a 1.5% agarose gel and the amplicons visualized after staining with ethidium bromide.

Induction and Measurement of Cytokine Release by TCCs

For analysis of cytokine production, quiescent T cells were washed three times in RPMI-1640 plus 5% FCS and restimulated with a mitogenic pair of mAbs directed against CD2 (clone CLB-11.1/1 and CLB-T11.2/1; CLB, Amsterdam, The Netherlands) in combination with an mAb directed against CD28 (clone CLB-28/1; CLB) in culture medium in 96-well tissue culture plates at 2×10^5 cells per well. This method has been shown to yield high cytokine production.¹⁷ The amounts of IL-4 and IFN- γ secreted in the supernatants, collected after 24 hours, were determined by solid-phase sandwich ELISA, as described previously.¹⁷ If the production of IL-4 was less than 10% of the production of IFN- γ , the cytokine pattern was arbitrarily classified as Th type 1. Similarly, if the production of IFN- γ was less than 10% of the production of IL-4, the cytokine pattern was classified as Th type 2. The cytokine pattern was classified as Th type 0 in case of intermediate mixtures of IFN- γ and IL-4 secretion levels.

RESULTS

Generation of TCLs from VF Samples of Patients with Recurrent Ocular Toxoplasmosis

Diagnostic analyses were performed on VF samples, obtained for therapeutic or diagnostic purposes, from 10 patients clinically suspected as having recurrent ocular toxoplasmosis. Production of intraocular antibody to Tg was demonstrated in all patients. PCR analyses revealed the presence of Tg DNA in the VF samples of patients 2, 3, and 6 (Table 1). In the VF samples of patients 11 and 12, who had panuveitis and pars planitis, no evidence for local Tg-specific antibodies or Tg DNA could be detected (Table 1). On the basis of clinical and laboratory data, the ocular lesions of patients 1 to 10 were defined as recurrent ocular toxoplasmosis.

The VF-derived inflammatory cells were expanded by one round of nonspecific stimulation with PHA in the presence of IL-2 and γ -irradiated allogeneic feeder cells. The VF-derived TCLs were screened for reactivity to crude protein lysates of Tg and human retinas. Significant Tg-specific proliferative responses were detected only in the TCLs of the patients with recurrent ocular toxoplasmosis (Table 2; patients 1–10). The SI of the TCLs varied from 3.1, which is just above threshold, to 243.5 but did not correlate with disease activity, Goldmann-Witmer coefficient, or Tg DNA PCR results (Table 1). None of the TCLs tested showed reactivity to the human retinal extract (Table 2).

TABLE 2. Antigen-Specific Proliferative Responses of VF-Derived TCLs

Patient	[³ H]-Thymidine Incorporation		
	Medium	Tg	Retina
1	977 \pm 198	5,697 \pm 1,615	832 \pm 79
2	76 \pm 12	12,895 \pm 492	140 \pm 10
3	603 \pm 452	10,160 \pm 2,152	268 \pm 274
4	168 \pm 115	5,946 \pm 278	34 \pm 21
5	108 \pm 23	26,302 \pm 4,121	56 \pm 12
6	64 \pm 17	4,439 \pm 456	61 \pm 44
7	91 \pm 15	7,995 \pm 1,100	38 \pm 19
8	311 \pm 77	10,630 \pm 1,891	202 \pm 45
9	246 \pm 54	11,574 \pm 3,180	208 \pm 32
10	260 \pm 128	803 \pm 122	ND
11	154 \pm 8	122 \pm 19	ND
12	79 \pm 14	107 \pm 12	72 \pm 14

T cells were incubated with autologous PBMCs in the presence of medium, *T. gondii* (Tg), or human retina protein extract (Retina). Data are expressed as the mean counts per minute \pm SD of triplicate cultures. ND, not done.

Characterization of VF-Derived Tg-Specific TCCs

To analyze the intraocular Tg-specific T-cell response at the clonal level, the T cells of the VF-derived TCLs of patients 1, 4, and 8 were cloned representatively by limiting dilution. A substantial number of TCCs were Tg specific. Of the TCCs obtained from patients 1, 4, and 8, 65 (31%) of 208, 17 (74%) of 23, and 6 (14%) of 44 were Tg specific, respectively. In all three patients, none of the TCCs was reactive to human retinal extract (data not shown). The Tg-specific TCCs, which after four rounds of restimulation had produced sufficient numbers of cells, were subjected to further testing. They were characterized with respect to their phenotype, HLA-restriction of antigen recognition, and TCR V β usage (Table 3). All 23 Tg-specific TCCs were CD3⁺CD4⁺. Nineteen of 21 Tg-specific TCCs tested were restricted by HLA-DR, whereas two TCCs (one of patient 1 and one of patient 8) showed HLA-DP restriction. The TCR V β gene usage of the Tg-specific TCCs of patients 4 and 8 was diverse (not tested for patient 1).

The production of the cytokines IFN- γ and IL-4 by the Tg-specific TCCs was determined (Table 3). In patient 4, who underwent a diagnostic vitrectomy at the time of active inflammation, 4 of 10 intraocular TCCs had a Th2-like phenotype. In contrast, almost all intraocular TCCs from patient 8 (disease activity grade 2) and patient 1 (disease activity grade 1) were Th0-like cells. Both patients underwent a vitrectomy at a stage when ocular inflammation had almost resolved.

DISCUSSION

In the present study, we show that a reactivation of latent ocular toxoplasmosis is associated with an ocular infiltration of a polyclonal Tg-specific T-cell population. In contrast, retinal autoantigen-specific T cells could not be detected in the VF-derived TCLs. This does not exclude the possibility that they may be present in the eye, either at a very low frequency or at the earliest stages of the inflammation. However, the significant SI in response to Tg antigens of the VF-derived TCLs from all patients with recurrent ocular toxoplasmosis compared with two control patients, and the high frequency of VF-derived Tg-specific TCCs generated from three patients, suggest a predominant intraocular Tg-specific T-cell response in the patients analyzed. Different therapies, including systemic, periocular, and topical corticosteroids were administered to the patients at the time of vitreous sampling (Table 1). Al-

TABLE 3. Proliferative Responses, Phenotype, TCR V β Gene Usage, HLA-Restriction, and Cytokine Secretion by VF-Derived Tg-Specific TCCs

Patient	TCC	SI to Tg*	Phenotype	TCR V β †	HLA‡	IL-4§	IFN- γ §	Th Type#
1	TCC 1	10.4	CD4	ND	HLA-DR	0.73	6.45	Th0
	TCC 2	28.9	CD4	ND	HLA-DR	2.52	11.0	Th0
	TCC 3	68.8	CD4	ND	HLA-DR	30.0	22.8	Th0
	TCC 4	15.6	CD4	ND	HLA-DR	2.39	4.55	Th0
	TCC 5	11.8	CD4	ND	HLA-DP	2.34	3.49	Th0
	TCC 6	17.6	CD4	ND	HLA-DR	2.78	55.2	Th1
	TCC 7	10.9	CD4	ND	HLA-DR	2.32	4.27	Th0
	TCC 8	8.5	CD4	ND	HLA-DR	2.51	7.27	Th0
4	TCC 9	158.2	CD4	V β 3	HLA-DR	0.76	0.06	Th2
	TCC 10	7.0	CD4	V β 21.1-4	HLA-DR	0.94	3.03	Th0
	TCC 11	66.2	CD4	V β 2	HLA-DR	2.09	1.71	Th0
	TCC 12	52.1	CD4	V β 10	HLA-DR	4.21	1.87	Th0
	TCC 13	18.1	CD4	V β 5.1	HLA-DR	0.08	1.98	Th1
	TCC 14	42.1	CD4	V β 17	HLA-DR	3.60	2.04	Th0
	TCC 15	30.0	CD4	V β 2	HLA-DR	4.99	0.14	Th2
	TCC 16	6.2	CD4	V β 5.2-3	ND	0.82	0.05	Th2
	TCC 17	14.8	CD4	V β 5.2-3	HLA-DR	1.29	0.04	Th2
	TCC 18	36.3	CD4	ND	ND	2.56	3.34	Th0
8	TCC 19	23.5	CD4	V β 2	HLA-DR	4.00	22.2	Th0
	TCC 20	107.0	CD4	ND	HLA-DR	12.9	27.6	Th0
	TCC 21	15.5	CD4	ND	HLA-DR	5.80	28.7	Th0
	TCC 22	57	CD4	V β 18	HLA-DP	2.20	12.1	Th0
	TCC 23	87	CD4	V β 6.1-3	HLA-DR	12.4	12.4	Th0

* SI was determined to *Toxoplasma gondii* (Tg) compared with medium control in proliferation assays.

† T-cell receptor variable β gene (TCR V β) usage was determined by RT-PCR. ND, not done.

‡ The HLA restriction determinant of the TCCs was determined using HLA-DP, -DQ and -DR blocking monoclonal antibodies in Tg-specific proliferation assays.

§ Secretion levels of IL-4 and IFN- γ was determined in culture supernatant of Tg-stimulated TCC and are expressed in nanograms/milliliter.

Th-cell type was defined arbitrary by the ratio of IL-4 and IFN- γ production. Th1, IL-4 to IFN- γ = <10%; Th2, IFN- γ to IL-4 = <10%; Th0, cells secreted a mixture of IFN- γ and IL-4.

though these immunosuppressive treatments may have influenced the total number of T cells in the vitreous samples, they did not seem to affect the ability of the TCLs and TCCs, generated from the VF, to respond to Tg antigens (Tables 2, 3).

In mice, both CD4⁺ and CD8⁺ T cells have been described to play a protective role in acute and chronic Tg infection.³ Although the VF-derived TCLs contained CD8⁺ T cells (data not shown), none of the Tg-specific TCCs obtained from these lines had this phenotype. It is possible that the type of assay or nature of the Tg antigen used—that is, proliferation assays using exogenous antigen preparations, account for the recovery of CD4⁺ and not CD8⁺ Tg-specific TCCs in the present study. Nevertheless, a similar CD4⁺ predominance has been reported for Tg-specific human TCCs generated from peripheral blood,^{18–20} even when autologous PBMCs infected with live Tg tachyzoites were used to stimulate the TCCs.²¹ It has been suggested that in chronic infections, as in our patient group, CD4⁺ T cells predominate, whereas in recently infected patients the majority of Tg-specific T cells are CD8⁺ T cells.²²

T-cell derived cytokines have been demonstrated to be critical in determining the outcome of Tg infections.^{3,23} The Th1 cytokines IFN- γ ²⁴ and IL-2²⁵ are considered to mediate protective effects. Th2 cytokines, such as IL-4^{26,27} and IL-10,^{28,29} have been shown to be associated with progression of disease. Increased intraocular levels of both IFN- γ and IL-10 have previously been reported in patients with ocular toxoplasmosis.³⁰ As in leishmaniasis,³¹ the balance between these Th-cell subsets may determine the outcome of Tg infections.³² By analyzing the cytokine secretion profile of the Tg-specific VF-derived TCCs obtained from three patients, two observations can be made. First, it was observed that most clones displayed a Th0-Th2 phenotype. This deviates from the expected Th1-like response associated with peripheral infection.³ It is conceivable that the microenvironment of the eye induces T cells to shift from the detrimental Th1 phenotype

toward a less harmful Th0-Th2 phenotype. Second, the clones from patient 4 with active disease were mostly Th2 compared with the Th0 clones from the eyes of patients 1 and 8 who were in the recovery phase of the disease.

In conclusion, this study is the first to demonstrate the presence of a T-cell response specific for the inciting microorganism in VF samples of patients with active recurrent ocular toxoplasmosis. The functional characteristics of the Tg-specific T cells and their presence at the site of inflammation suggest their involvement in the local inflammatory response of ocular toxoplasmosis. An important question is whether Tg-specific T-cell responses have a beneficial or a deleterious effect. The immune-mediated damage to the retina may in fact have a more detrimental impact than the cytopathic effect of the parasite itself. Further characterization of their antigenic specificity and functional properties may have implications for future vaccine development against this blinding disease.

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