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Interferon-γ and IL-10 Release Assay for Patients with Ocular Toxoplasmosis

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Abstract. Peripheral blood mononuclear cells (PBMC) from patients with ocular toxoplasmosis were challenged with total antigens from *Toxoplasma gondii* lysate (TATL) in a cytokine release assay (CRA), run during the inactive period of the disease. Increased interferon gamma (IFN-γ) levels were detected after PBMC stimulation with either ME49 reference strain ($P = 0.0015$) or local TgCkAr-11-9 isolate ($P = 0.0012$), as compared with those recorded under basal conditions. TATL from TgCkAr11-9 isolate induced a higher release of IFN-γ than ME49 strain in CRA from all tested patients ($P = 0.02$). The median value of IFN-γ release on TgCkAr-11-9 stimulation (26.03 pg/mL) allowed the classification of patients into high– or low–/non–IFN-γ releasers. Clinical correlations were established with both groups. The results obtained in this study suggest the need to include local strains when performing CRA with TATL.

INTRODUCTION

Ocular toxoplasmosis (OT) is the main cause of posterior uveitis worldwide and is a consequence of infection by the zoonotic Apicomplexan protozoan *Toxoplasma gondii*. In South America, OT is extremely frequent in the Eastern region (ER) of the 27°S parallel (Southern Brazil and Misiones in the northeast of Argentina)[1,2] as well as in Colombia.[3] Severe atypical OT presentations such as bilateral active ocular retinochoroiditis in young immunocompetent patients and vertical transmission of the disease during OT reactivation were described in the ER of the 27°S parallel.[4,5]

One of the potential reasons believed to be related to the high frequency and severity of OT in South America is the genetic divergence of *T. gondii* strains isolated in the aforementioned regions. A phylogenetic analysis of *T. gondii* population structure in Argentina demonstrated a differential genotype pattern between the center (Buenos Aires Province) and the northeast of the country (Misiones Province). Whereas *T. gondii* isolates from the center of Argentina were closely related to a type III reference strain, the isolates from Misiones were grouped between type I and type III reference strains, being associated with isolates from Brazil.[6,7] In Southern Brazil, a rate of OT five times higher than in Europe has been reported and is suggested to be associated with atypical genotype infection.[8]

Approximately 80% of OT patients living in Misiones will seek ophthalmic consultation when they already have toxoplasmic ocular scars associated with an area of active retinochoroiditis (reactivation of toxoplasmic retinochoroiditis [RTR]). Primary toxoplasmic retinochoroiditis is less frequently detected. Persistent inflammation of the vitreous humor (vitritis) despite an appropriate medical treatment is a complication observed in patients with RTR in South America. Persistent vitritis (PV) has been previously associated with low intraocular interferon gamma (IFN-γ) production, an IFN-γ/interleukin-10 (IL-10) ratio lower than 1 in aqueous humor (AH), as well as with severe ocular complications such as retinal detachment (RT).[9]

The development of a noninvasive methodology capable of the identification of patients with low intraocular IFN-γ production is crucially important because it may allow the identification of OT patients at risk during RTR. The release of IFN-γ by peripheral blood mononuclear cells (PBMC) has been used to confirm tuberculosis and congenital toxoplasmosis infection of newborn suspected patients.[10] In France, the IFN-γ release assay test was used for the diagnosis of congenital toxoplasmosis, exhibiting 94% of sensitivity and 98% of specificity.[11,12]

We decided to explore the possible association between IFN-γ and IL-10 levels obtained in a cytokine release assay (CRA)[13] performed during the inactive period of OT disease and clinical signs from the same patients during their last RTR. Furthermore, in a subset of these patients, we compared their previously known AH IFN-γ levels analyzed during RTR versus IFN-γ levels released in the CRA.

PATIENTS, MATERIALS, AND METHODS

All patients suffering uveitis were examined by a single ophthalmologist, specialized in such disease (M. R.), in a private secondary care eye clinic in Oberá, Misiones Province, Argentina. M. R. performed complete ophthalmological examinations, including visual acuity, anterior biomicroscopy, tonometry, and indirect ophthalmoscopy. Reactivation of RTR was defined as a white yellowish elevated area of the retina near or at the border of a pigmented retinochoroidal scar with vitritis and vasculitis in patients with reactive serology against *T. gondii*. Ocular hypertension (OH) was defined as intraocular pressure higher than 21 mm of Hg. Control individuals (CI) proved negative for anti–*T. gondii* antibodies in the absence of ocular signs of toxoplasmosis. Results from 14 OT patients were analyzed, including nine patients whose AH IFN-γ and IL-10 results were previously known. Cytokine release assay was performed in the Research Center of the Universidad Católica

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de las Misiones. Blood samples for the CRA were drawn from the patients during the inactive cicatricial scar period of the disease (elapsed time after RTR ranged between 38 and 164 weeks, average time was 102.71 ± 39.85 weeks, and median time was 97 weeks). Peripheral blood mononuclear cells were purified using Histopaque-1077 following the manufacturer's instructions (Sigma-Aldrich [currently Merck KGaA, Darmstadt, Germany]). The cells were washed three times with Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma-Aldrich) supplemented media. Then, cells (0.4×10^6) were plated in 300 µL/well onto a 96-well plate. Peripheral blood mononuclear cells were stimulated with either 1 µg/mL of phytohemagglutinin (PHA) or the total antigens from *T. gondii* lysates (TATL). The TATL from two dissimilar strains—clonal type II ME49 reference strain and non-clonal TgCkAr-11-9 local isolate—was tested.[6,7] Cells were incubated at 37°C in a 5% CO_2 atmosphere. The supernatants were obtained after 24 hours of incubation. The number of cells plated onto each well, the concentration of the TATL or PHA (0.5–3 µg), and the incubation period (12, 24, or 48 hours: second being the most appropriate) were settled after experimental optimization. Interferon gamma and IL-10 levels released in the CRA supernatant were measured by ELISA (ThermoFisher, Pierce Biotechnology, Rockford, IL). The cutoff value for ELISA was calculated by adding three SDs to the average of basal control conditions. Therefore, the IFN-γ cutoff value was determined at 6.7 pg/mL.

The Wilcoxon signed-rank test was used to compare the values of IFN-γ released by PBMC under basal conditions, as compared with those recorded on stimulation with the TATL from ME49 or TgCkAr-11-9. The same test was used to compare IFN-γ results obtained by ME49 or TgCkAr-11-9 stimulation. The median of IFN-γ results obtained after PBMC stimulation with TgCkAr-11-9 (26.03 pg/mL) was used as a cutoff value to allocate patients within one of two groups: those with IFN-γ results above the median were considered as high IFN-γ releasers, whereas those below the median were regarded as low IFN-γ releasers, including a subgroup of non–IFN-γ releasers. The Mann–Whitney test was used to analyze differences in IFN-γ/IL-10 ratio observed between the aforementioned IFN-γ release groups. Fisher's test was used to compare proportions of patients with specific clinical ophthalmological signs, such as OH, between IFN-γ release groups. The Spearman coefficient was used to analyze correlations between IFN-γ results released by PBMC upon TgCkAR-11-9 stimulation with ocular tension and with AH IFN-γ levels measured during the last RTR episode. A P-value < 0.05 was considered statistically significant. Analysis was performed using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY).

RESULTS

Basal levels of IFN-γ released by PBMC in the CRA ranged from 3.23 to 6.19 pg/mL (median = 4.92 pg/mL) in patients and from 3.31 to 5.23 (median = 4.21 pg/mL) in CI (see Table 1). When compared with basal PBMC IFN-γ levels, a significant increase in the release of IFN-γ was observed upon PBMC stimulation with either TATL from ME49 (P = 0.0015) or TATL from TgCkAr-11-9 (P = 0.0012) in patients (see Figure 1). TATL from TgCkAr11-9 local isolate induced the release of higher IFN-γ levels (median = 26.03 pg/mL) than did ME49 reference strain (median = 12.88 pg/mL) by PBMC in the CRA from all tested patients (P = 0.02).

The group of high IFN-γ releasers after PBMC stimulation with TgCkAr-11-9 (patients #1–3, #7, #9, #12, and #13) exhibited an IFN-γ/IL-10 ratio higher than 1. By contrast, low IFN-γ releasers (patients #4–6, #10, and #11) and non–IFN-γ releasers (with IFN-γ values below the ELISA cutoff level, e.g., patients #8 and #14) exhibited a score ratio < 1. The difference between both groups (high and low IFN-γ releasers) was statistically significant (P = 0.0017). Five of seven patients with high IFN-γ release levels in the CRA and none of seven with low or non–IFN-γ release exhibited OH during RTR (P = 0.010). A positive correlation between released IFN-γ in the CRA and intraocular pressure was observed (r = 0.943; P < 0.001). Furthermore, another correlation between IFN-γ released upon PBMC stimulation with TgCkAr-11-9 and AH IFN-γ levels analyzed during the last RTR episode was observed, although it failed to reach statistical significance (r = 0.66; P = 0.0525).

Patients #8 and #14 had experienced a congenital *T. gondii* infection and had no IFN-γ release upon stimulation with either ME49 or TgCkAr-11-9. Patients #6 and #8 corresponded to a very low IFN-γ releaser and to a non–IFN-γ releaser, respectively. Both patients experienced an evolution period longer than 10 weeks to resolve their RTR.

DISCUSSION

The production of IFN-γ by human's PBMC against intracellular pathogens such as *T. gondii* is not only genetically determined by polymorphisms of the IFN-γ gene[14,15] but also by the gene polymorphisms of molecules capable to inhibit IFN-γ synthesis, such as IL-10.[15,16] Interferon gamma production is also altered by DNA methylation and post-transcriptional mechanisms, such as miRNAs modification of IFN-γ mRNA.[17] In addition, the release of IFN-γ by human PBMC after the challenge with *T. gondii* may be affected by the type of infection (either congenital or acquired)[18] and by the presence or absence of ocular or cerebral lesions.[19]

In the current study, we performed a CRA to quantify the release of IFN-γ and IL-10 upon the challenge of patient's PBMC with TATL from two dissimilar *T. gondii* strains. We also compared the levels of released IFN-γ in the CRA by patients in the inactive cicatricial period against the clinical ophthalmic signs and values of IFN-γ obtained during RTR. Although the number of patients from whom the CRA could be carried out is still limited (n = 14), we were able to draw some interesting initial conclusions, as follows:

First, higher IFN-γ levels were released in the CRA when using TATL from TgCkAr-11-9 local isolate than from ME49 reference strain as antigenic stimuli for all OT patients residing in Misiones Province, a result that might reflect the predominant circulation of the former strain in this area, thus possibly influencing the course of the infection.

Second, the corroboration that patients with high intraocular IFN-γ during RTR also release high levels of IFN-γ in the CRA during the inactive period of the ocular disease. As we have previously shown,[9] this group of patients experienced high intraocular pressure during RTR. We observed a positive correlation between IFN-γ released in the CRA and such clinical sign.

Third, CRA was a useful diagnostic tool to show either the absence or very low levels of released IFN-γ. This group included patients who exhibited either a congenital[17] or an acquired *T. gondii* disease, both of them with PV and a longer elapsed time to RTR resolution.

TABLE 1

Clinical data, serum anti–*T. gondii* IgG antibodies, IFN-γ, IL-10 levels in addition to IFN-γ/IL-10 ratio obtained from the supernatant of a CRA, and intraocular IFN-γ values obtained during RTR

					Inactive disease				Active disease					
					CRA									
Individual	Gender	Age (years)	Toxoplasmosis	Toxo IgG IU/mL	IFN-γ (pg/mL) from PBMC	IFN-γ (pg/mL) from PBMC + ME49 *	IFN-γ (pg/mL) from PBMC + TgCkAr-11-9** ***	IFN-γ/IL-10 ratio#	Intraocular aqueous humor IFN-γ pg/mL (during RTR)	Clinical signs	Macular involvement	RTR duration (weeks)	Complications	Active lesion size (papillary diameter)
P1	F	66	Acquired	80.99	4.75	25.07	122.3	4.42	135	OH, seven RTR episodes	No	6	EM	1.5
P2	M	42	Acquired	135.54	5.95	17.81	42.58	2.07	105.6	OH, RV	No	6	Cataract	2
P3	M	29	Acquired	156.49	6.07	132.40	291.57	3.54	64	OH, RV	Yes	6	–	2.5
P7	M	20	Acquired	134.72	6.19	87.36	233.9	8.98	15	SF	Yes	8	–	1
P9	M	27	Acquired	138.24	5.27	833	1,000	8.4	732.23	OH, RV	No	7	–	–
P12	F	46	Acquired	141.3	4.25	682	1,000	9.6	–	OH	Yes	7	–	–
P13	M	37	Acquired	1,024†	4.68	26.3	37.6	1.2	–	RV	No	6	EM	–
P4	M	39	Unknown	178.96	4.27	4.99	12.06	0.19	17.9	–	Yes	9	Neutropenia	2
P5	M	16	Congenital	211.017	5.32	6.78	13.49	0.17	17.9	Concomitant bilateral inflammation	Yes	8	–	3
P6	M	60	Acquired	161.76	4.75	6.19	7.6	0.13	17.7	Successive bilateral inflammation, PV	No	> 10	RD	–
P10	F	38	Acquired	125.84	3.23	7.95	14.47	0.6	–	Yuxtapapilar lesion	No	7	–	2.5
P11	M	30	Acquired	138.5	3.93	6.72	10.44	0.87	–	–	Yes	8	–	1.5
P8	M	24	Congenital	512†	5.71	4.99	5.23	0.08	2	MRL, PV	No	> 10	Cataract	2
P14	F	39	Congenital	125.33	5.1	5.9	6.24	0.1	–	MRL	Yes	8	–	2
CI1	F	35	–	Neg	5.23	3.31	4.51	0.11	–	–	–	–	–	–
CI2	F	38	–	Neg	4.51	5.2	4.27	0.05	–	–	–	–	–	–
CI3	M	48	–	Neg	3.7	4.26	4.075	0.09	–	–	–	–	–	–
CI4	F	21	–	Neg	3.31	–	3.02	–	–	–	–	–	–	–

CI = control individual; CRA = cytokine release assay; EM = epiretinal membrane; F = female; IFN-γ = interferon gamma; M = male; MRL = multiple retinal lesion; OH = ocular hypertension; PBMC = peripheral blood mononuclear cells; PV = persistent vitritis; RTR = reactivation of toxoplasmic retinochoroiditis; RV = retinal vasculitis; SF = subretinal fibrosis; RD = retinal detachment; *T. gondii* = *Toxoplasma gondii*; Neg = negative. Cytokine release assay was performed during the inactive scar period. Peripheral blood mononuclear cells from blood of four anti–*T. gondii* seronegative subjects were used as CIs for CRA. A significant increase in the release of IFN-γ was observed upon patients' PBMC stimulation with total antigens from *Toxoplasma gondii* lysate (TATL) from ME49 reference strain (*$P = 0.0015$) or from TgCkAr-11-9 (**$P = 0.0012$) local isolate. TATL from TgCkAr11-9 induced the PBMC release of higher IFN-γ levels (median = 26.03 pg/mL) than ME49 (median = 12.88) in CRA from all tested patients (***$P = 0.02$). Patients with IFN-γ results above the median were considered high IFN-γ releasers and have an IFN-γ/IL-10 ratio > 1. A significant difference was observed between patients with IFN-γ/IL-10 ratios > 1 vs. patients with such ratio < 1 (#$P = 0.0017$).

† = antibody titer obtained by an indirect hemagglutination test.

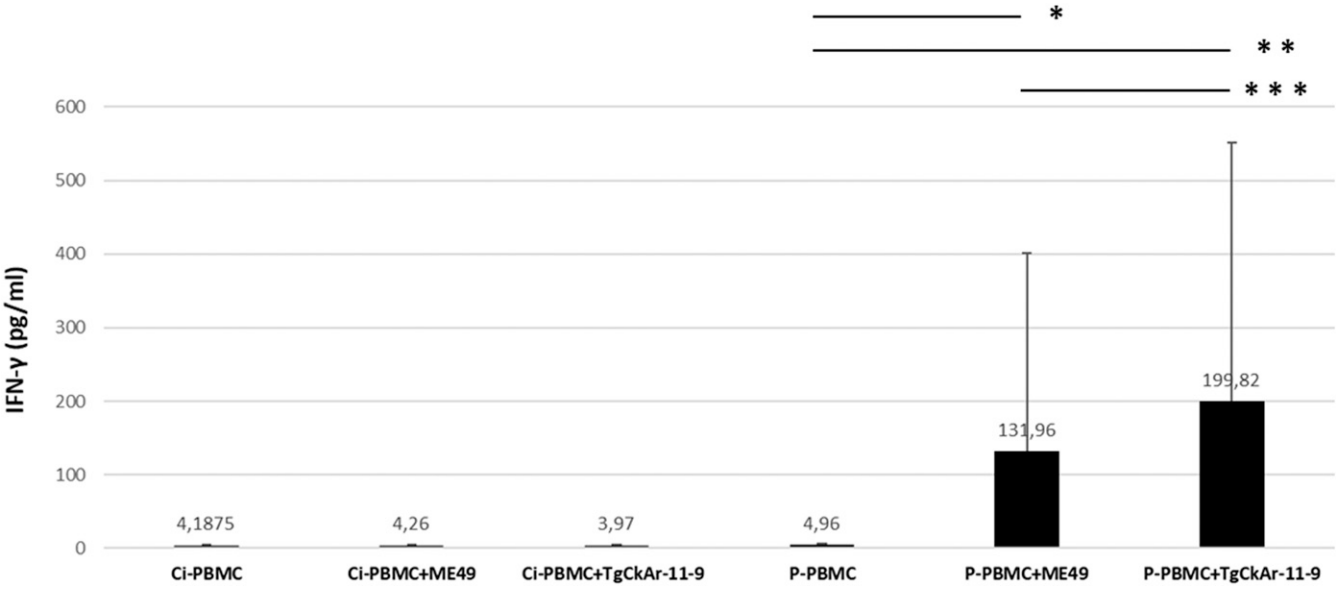

FIGURE 1. Interferon gamma levels released from peripheral blood mononuclear cells (PBMC) obtained from control individuals (CI) and patients (P), under basal conditions (CI-PBMC and P-PBMC) and upon stimulation with TATL from either ME49 reference strain or TgCkAr-11-9 local isolate, as registered in CRA are shown. A significant increase in the release of IFN-γ was observed upon P-PBMC stimulation with TATL from either ME49 (*P = 0.0015) or TgCkAr-11-9 (**P = 0.0012). TATL from TgCkAr11-9 local isolate induced a higher P-PBMC release of IFN-γ levels (median = 26.03 pg/mL) than ME49 reference strain (median = 12.88) in CRA from all tested patients (***P = 0.02). CRA = cytokine release assay; IFN-γ = interferon gamma.

In conclusion, patients with OH producing high intraocular IFN-γ levels during RTR can be identified as high releasers of IFN-γ by PBMC in CRA. Cytokine release assay is a simple and useful test that may allow the identification of patients with acquired OT, exhibiting low release of IFN-γ. The latter group of patients is associated with an increased probability to experience a longer time of evolution and severe complications. The results obtained in this study suggest the need to include local strains when performing CRA with TATL.

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