Discrimination between Patients with Acquired Toxoplasmosis and Congenital Toxoplasmosis on the Basis of the Immune Response to Parasite Antigens

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Many persons infected with *Toxoplasma gondii* develop ocular lesions. Immunologic parameters in the response to *T. gondii* were evaluated in infected persons with and without ocular lesions and in noninfected controls. Subjects were divided into groups on the basis of presence of serum antibodies to *T. gondii,* presence of ocular lesions, and clinical history. Production of interleukin-2 and interferon-γ by peripheral blood mononuclear cells from patients with probable congenital toxoplasmosis was decreased, compared with that in persons with presumed acquired infection. Cell proliferation and delayed-type skin reaction induced by soluble toxoplasma tachyzoite antigen followed the same pattern. Asymptomatic persons showed high levels of interleukin-12 and interferon-γ, whereas persons with ocular lesions had high interleukin-1 and tumor necrosis factor-α responses toward soluble toxoplasma tachyzoite antigen. These data suggest that patients with ocular disease due to congenital infection show tolerance toward the parasite. Furthermore, susceptibility to ocular lesions after acquired toxoplasmosis is associated with high levels of interleukin-1 and tumor necrosis factor-α, whereas resistance is associated with high levels of interleukin-12 and interferon-γ.

Toxoplasmosis is caused by the intracellular protozoan *Toxoplasma gondii.* Acquired disease may result in lymphadenopathy, low-grade fever, and sore throat. In general, the disease progresses to a symptomless state in immunocompetent persons [1]. Disease is often fatal in the immunocompromised host, mostly because of encephalitis [2–5]. Ocular lesions are caused by either congenital or acquired infection. In the United States, infection occurs in 2/1000 pregnancies, with a transplacental infection rate ⩽50% [6]. Seventy percent of infants with congenital infection show chorioretinal scars. Acquired disease was thought to be rare [7, 8]. However, several studies indicate that ocular lesions may be caused by *T. gondii* infection after birth [1, 9–13]. Ocular toxoplasmosis is characterized by a necrotizing retinitis with oval or circular lesions. Reactivation sites usually appear in proximity to older atrophic lesions [8, 14, 15].

Recent clinical and epidemiologic studies indicate that 95% of the population of Erechim, a region in southern Brazil, are seropositive for *T. gondii* [1, 10]. Interestingly, 20% of Erechim's population develop ocular toxoplasmosis, most of which is thought to be acquired [1, 10]. This population therefore offers a unique opportunity to study the differences in the immune response to toxoplasma antigens between infected persons with or without ocular lesions and in persons with congenital disease. We sought to distinguish between patients with congenital toxoplasmosis and those with acquired disease on the basis of their lymphokine response toward *T. gondii*. We therefore studied resistance and susceptibility to the development of ocular lesions in relation to differences in the patients' cytokine profiles.

Methods

Patients. Blood samples were collected from 136 subjects 18–64 years old; sexes were equally represented. Twenty-six subjects had

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Blood was drawn after informed consent was obtained from each subject. All procedures were approved by the ethics panels of the institutions (Universidade Federal de São Paulo, University of São Paulo) at which the patients were being followed and by the internal review board of the National Eye Institute, National Institutes of Health.

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negative titers of antibody (IgM and IgG) to *T. gondii* (titer, <1 : 4). All others had positive titers of antibody to toxoplasma (IgM or IgG; >1 : 64). Antibody titers were evaluated by a test kit (Sigma, St. Louis). Subjects came from Erechim or São Paulo, Brazil. Clinical status (presumed acquired ocular toxoplasmosis, congenital ocular toxoplasmosis, or absence of ocular lesions) was determined, as described elsewhere [1, 9, 10]. In brief, patients were presumed to have acquired toxoplasmosis if they had had a funduscopic examination with a clear fundus before the visit in which disease was diagnosed, if their mother had a positive titer of IgG antibody before pregnancy, or if they had an older sibling with documented ocular toxoplasmosis. Patients were presumed to have congenital toxoplasmosis leading to ocular lesions if their mother had a positive IgM response to toxoplasma antigens during pregnancy and if, at their first visit to the ophthalmologist, ocular lesions were visualized at the funduscopic examination.

Of note, all patients with probable congenital toxoplasmosis studied had ocular lesions. No subject was receiving treatment with any immunosuppressive or immunomodulatory drug. Women who used oral contraceptives were not included in this study, because these drugs may alter the antigen-specific response patterns of T cells (authors' unpublished data). Other drugs being used by the patients during the time of the study included antibiotics (2 patients), aspirin (2 patients), acetaminophen (3 patients), and antihistamine (5 patients).

Proliferation assays. Proliferation assays were done as described elsewhere [16]. In brief, peripheral blood mononuclear cells (PBMCs), obtained by gradient centrifugation, were diluted to 10^6 cells/mL and added to 96-well flat-bottom microtiter plates (Falcon, Oxnard, CA). Cultures were stimulated with either 2.5 μg/mL phytohemagglutinin (PHA) or 5 μg/mL soluble toxoplasma tachyzoite antigen (STAg) or control antigens (tuberculin purified protein derivative [PPD] or tetanus toxoid), in a final volume of 200 μL of RPMI 1640 per well, containing 5% AB^+ pooled human serum. PBMCs were cultured for 96 h and then pulsed with 0.5 μCi/well [^{3}H]thymidine and processed accordingly for standard gaseous or liquid scintigraphy. Results are presented in stimulation index (SI) units that represent the mean proliferation in counts per minute to a given stimulus divided by the mean background proliferation of unstimulated cells. SIs >2 were considered positive. PBMCs were obtained from blood drawn >1 month after the last active episode of disease and ≤2 months after the end of that episode.

Preparation of STAg. STAg was prepared as described elsewhere [17]. In brief, tachyzoites of the RH strain were maintained by in vitro passage in human foreskin fibroblasts at 37°C. For antigen preparation, tachyzoites were harvested from fibroblast cultures, passed through a 27-gauge needle, centrifuged at 70 *g* for 5 min, and pelleted at 590 *g* for 10 min. Tachyzoites were then sonicated 4 times for 20 s each round and centrifuged at 10,000 *g* for 30 min. The supernatant preparation, termed STAg, was used as antigen in the in vitro assays.

Interleukin (IL) measurement. Human IL-2 was measured by the proliferation of the murine indicator T cell line HT-2 or by ELISA with paired antibody (PharMingen, San Diego). IL-1, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)–α, and interferon (IFN)–γ were also measured by ELISA as described elsewhere [16].

Flow cytometry. Standard flow cytometry analysis was done with antibodies to human V_α and V_β chain (Endogen, Cambridge, MA; or PharMingen). One million cells were stained directly in a final volume of 100 μL of PBS, according to the manufacturer's instructions, and were read (FACScan with Lysis II software or FACScallibur with CellQuest software; Becton Dickinson Immunocytometry Systems, San Jose, CA).

Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of V_β T cell receptor (TCR) chain. Analysis of the TCR by RT-PCR was done as described elsewhere [18]. RNA from PBMCs was obtained by the RNAzol B method, reverse-transcribed, and amplified by use of primers described elsewhere [18]. PCR products were separated on 1% agarose gels and analyzed by Southern blot hybridization with fluorescein-labeled internal probes, using the ECL-3′ oligolabeling and detection system and Hyperfilm (Amersham, Buckinghamshire, UK).

Reagents. Ficoll-hypaque was obtained from Sigma; PHA was obtained from Difco (Detroit). IL-2 was purchased from Boehinger (Mannheim, Germany), human IL-4 and IFN-γ were purchased from Genzyme (Cambridge, MA); human IL-1, IL-12, and TNF-α were obtained from R&D Systems (Minneapolis); and human IL-10 and IL-5 were from PharMingen.

Statistical analysis. The significance of the data was determined by analysis of variance, Fisher's paired least-square difference, and the Kruskal-Wallis test for nonparametric data. Results were considered statistically significant when a 95% confidence level was achieved.

Results

In a recent population-based household survey in Erechim, 184 (17.7%) of 1042 persons examined were considered to have ocular toxoplasmosis [1, 3, 6, 9]. Because of the high frequency of the disease [1, 3, 11], its occurrence in multiple siblings [1], and its low prevalence in children, compared with individuals >13 years old [10], many of the ocular toxoplasmosis cases in Erechim are thought to be sequelae of postnatal infection. This population represents an ideal setting for the study of the differences in the immune response between persons with acquired and congenital disease, as well as the immune mechanisms related to resistance and susceptibility to the development of ocular lesions. Patients who have been followed by some of us (C.S. and R.B.) were grouped as follows: normal controls with negative serology for toxoplasma, age- and sex-matched with study subjects, patients with positive serology for *T. gondii* but without ocular lesions, patients with a diagnosis of acquired ocular toxoplasmosis, and patients with a diagnosis of congenital ocular toxoplasmosis.

In vitro immune response to toxoplasma antigens. Figure 1 shows the stimulation indices obtained when PBMCs from the different groups were cultured in the presence of STAg. Although some seronegative persons had positive proliferative responses (SI ⩾2), most persons from that group did not respond to toxoplasma antigens. In contrast, PBMCs from seropositive persons, irrespective of the presence of ocular lesions, proliferated significantly in response to STAg. Nevertheless,

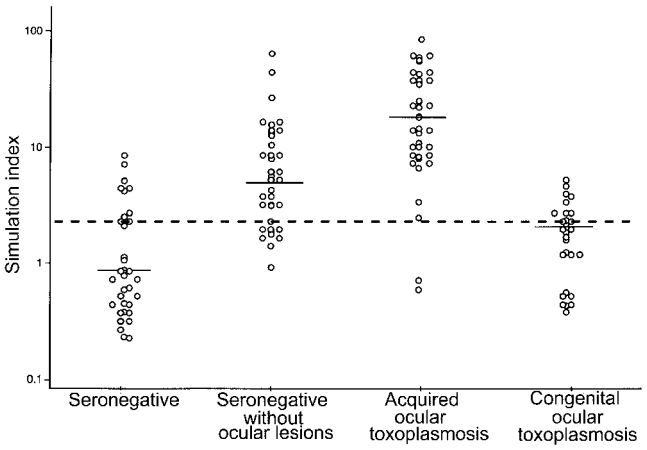

Figure 1. Peripheral blood mononuclear cell proliferation to soluble toxoplasma tachyzoite antigen. Dashed line indicates limit above which stimulation indices (SIs) are considered positive (control median + SD, 0.9 + 2.0). Horizontal line for each group represents median SI for that group: 0.9, 5.4, 18, and 2.3 for seronegative (n = 26), seropositive without ocular lesions (n = 40), presumed acquired ocular toxoplasmosis (n = 40), and probable congenital toxoplasmosis (n = 30), respectively. Average SI to phytohemagglutinin was 29.8, 25.6, 21.0, and 30.1, respectively. Proliferation was measured at 48 h, and antigen-specific proliferation was measured at 96 h. Average SI to tuberculin purified protein derivative was 10.6, 11.9, 13.7, and 12.1, respectively. Average SI to tetanus toxoid was 9.9, 8.7, 8.5, and 10.0, respectively.

persons with a diagnosis of acquired ocular toxoplasmosis had a median SI of 18, significantly higher than that of asymptomatic persons (SI, 5.4). Interestingly, patients with congenital toxoplasmosis had significantly lower SIs (median, 2.3) than did those with acquired disease. There was no difference between the groups in response to PHA or unrelated antigens (PPD and tetanus toxoid). Some patients from each group were analyzed for skin delayed-type hypersensitivity response to toxoplasma antigens, according to a protocol described elsewhere [19]. Patients with acquired ocular toxoplasmosis showed the strongest skin delayed-type hypersensitivity reaction elicited by *T. gondii* antigens. In contrast, patients with congenital ocular disease had no reaction to toxoplasma antigen in vivo (data not shown). Because of the small number of patients whose delayed-type hypersensitivity response was evaluated, the differences between groups were not statistically significant. Nevertheless, the finding that the in vitro PBMCs proliferation in response to toxoplasma antigens differs between the group with acquired disease and the group with congenital disease suggests that these two different forms of disease, although clinically indistinguishable, can be differentiated by a laboratory test. In addition, it suggests that the immune response to parasite antigens differs, depending on when the infection was acquired. To further study this possibility, we analyzed the patterns of cytokine response to toxoplasma antigens in the same groups described above.

Cytokine profile in response to toxoplasma antigens. Patients with a diagnosis of acquired ocular toxoplasmosis had significantly higher levels of IL-1 ($P < .05$) than did asymptomatic persons (table 1). TNF-α and IL-10 secretion in response to STAg were also increased in patients with acquired disease although the differences were not statistically significant (table 1). On the other hand, asymptomatic persons secreted significantly more IL-12 ($P < .05$) than did patients with acquired lesions. IFN-γ levels also were higher in asymptomatic persons than in patients with acquired toxoplasmosis. However, the difference was not statistically significant. IL-4 and IL-5 were undetectable in all groups in response to STAg although both cytokines could be detected in the supernatants from patient PBMCs stimulated with at least one of the control antigens. In keeping with the lymphocyte proliferative responses, lymphocyte-derived cytokine production in response to STAg was lower in PBMCs from patients with congenital toxoplasmosis than in patients with acquired disease. Taken together, these data suggest that patients with congenital disease develop a degree of tolerance toward toxoplasma antigens. Whether this tolerance is due to negative selection in the thymus or to a peripheral mechanism is a question currently under investigation in our laboratory. In addition, lymphocyte-derived cytokines, such as IL-2 and IFN-γ, are diminished in patients with congenital disease in response to STAg, whereas macrophage-derived cytokines, such as IL-1 and TNF-α, are overexpressed, suggesting that different mechanisms may be involved in the development of the ocular lesions. If indeed the mechanisms underlying the development of chorioretinitis in patients with acquired disease are different from those in patients with congenital disease, the therapeutic approach to these lesions should also differ although the histopathologic characteristics and their appearance on funduscopic examination are the same.

Because PBMCs from persons with congenital toxoplasmosis

Table 1. Cytokine production in response to soluble toxoplasma tachyzoite antigen among patients with acquired or congenital toxoplasmosis.

Cytokine	Seronegative (n = 26)	Seropositive (n = 40)	Acquired (n = 40)	Congenital (n = 30)
IFN-γ	BL	7.96 (2.48)	2.27 (1.24)	BL
TNF-α	0.86 (0.12)	2.9 (0.5)	8.77 (0.93)	6.02 (0.84)
IL-1	0.54 (0.09)	1.35 (0.4)	10.61 (4.8)[a]	5.56 (1.24)
IL-2	BL	0.33 (0.12)	0.49 (0.13)	BL
IL-4	BL	BL	BL	BL
IL-5	BL	BL	BL	BL
IL-10	BL	0.05 (0.02)	0.09 (0.02)	BL
IL-12	0.1 (0.01)	0.64 (0.08)[b]	0.22 (0.05)	0.26 (0.04)

NOTE. Data are ng/mL (SE). BL, below detection limit for assay. Detection limits (expressed as pg/mL) were as follows: interferon-γ (IFN-γ), 10; tumor necrosis factor-α (TNF-α), 20; interleukin-1 (IL-1), 40; IL-2, 10; IL-4, 50; IL-5, 20; IL-10, 50; IL-12 (p70), 20. Supernatants from cells obtained from the same patients and stimulated with phytohemagglutinin, tuberculin purified protein derivative, or tetanus toxoid showed cytokine levels above the detection limit, confirming that assays were adequate.

[a] Significantly higher ($P < .05$) than among seropositive asymptomatic patients.

[b] Significantly higher ($P < .05$) than among patients with acquired disease.

showed a degree of hyporesponsiveness in vivo and in vitro, we addressed the possibility that in these persons, T cells that respond to toxoplasma antigens were deleted early during ontogeny. Therefore, PBMCs from patients with congenital toxoplasmosis were analyzed for deletions of specific V_β-bearing lymphocytes. The presence of V_β families was tested by flow cytometry, when antibodies were available, or by RT-PCR, for the V_β families against which antibodies are not available. Deletions of V_β3, V_β12, V_β14, and V_β15 were observed. One patient had a deletion of V_β5.1 cells to <0.5% by flow cytometry (data not shown). However, some patients did not show any gaps in their V_β repertoire. The V_β deletions found in patients with congenital toxoplasmosis were often shared by members of the same family, suggesting that these were familial variations that were not due to the nature of the *Toxoplasma* infection. This last finding is particularly important, because if specific T cells are not deleted in patients with congenital toxoplasmosis, another mechanism must account for the deficient immune response they show against STAg. It is still possible that deletion of STAg-specific T cells occurs and that the methods used to evaluate TCR V_α and V_β families are not sufficiently sensitive to detect minor changes in these families. However, we must consider that other mechanisms may be responsible for the decrease in proliferation and cytokine production seen in the PBMCs from patients with congenital toxoplasmosis. Studies are under way in our laboratory to investigate some of the possible mechanisms involved.

TCR V_β expression. Studies in mice have suggested that *T. gondii* contains a superantigen-like activity capable of selective induction of T cells bearing the V_β5 chain both in vitro and in vivo [20–22]. Because superantigen stimulation causes expansion and ultimately deletion of the T cells bearing the TCRs with which they interact [20–22], we hypothesized that if *T. gondii* expresses a superantigen, exposure to STAg would result in the expansion of T cells bearing specific V_β chains in seronegative persons. Therefore, we stimulated PBMCs from seronegative persons with STAg. Five days after stimulation, the V_α and V_β repertoire in culture was evaluated by flow cytometry in comparison with the V_α and V_β repertoire expressed by unstimulated cells. A V_α or V_β was considered expanded if the percentage of positive cells after stimulation was ≥2-fold higher than the frequency in the unstimulated population. Nine of 26 seronegative persons tested showed an expansion of some T cells bearing the V_β5.1 receptor (table 2). Increases in the percentage of T cells expressing V_β5.2/V_β5.3, V_β6.7, V_β7.1, V_β8, V_β9, and V_β23 were also found. One patient showed a significant expansion of V_α12.1-bearing cells. The fact that T cells from unexposed persons proliferate in response to STAg supports the hypothesis that a superantigen expressed by *T. gondii* is capable of stimulating human T cells. This hypothesis is further supported by the finding that such proliferation is made through the expansion of specific V_β-bearing T cells. Interestingly, one of the V_β families that is found expanded in patients after stimulation with STAg is the same as the one seen expanded in response to *Toxoplasma* stimulation in the mouse [21, 22]. However, we found no evidence of specific V_β family expansion in ex vivo PBMCs from persons with congenital ocular toxoplasmosis or acquired ocular toxoplasmosis or from asymptomatic seropositive persons.

Table 2. V_β expression in peripheral blood mononuclear cells from seronegative subjects after stimulation with soluble toxoplasma tachyzoite antigen.

Patient	V_β enhanced
1	V_β5.1 (50)
2	V_β5.2/V_β5.3 (150), V_β9 (148)
3	None
4	None
5	V_β5.1 (200)
6	None
7	V_β5.1 (210), V_β8 (167)
8	None
9	V_β5.1 (202), V_β7 (135)
10	V_β5.1 (154), V_β6.7 (195)
11	V_α12.1 (149)
12	None
13	V_β5.1 (102), V_β5.2/V_β5.3 (183 and 190)
14	V_β9 (163)
15	V_β23 (102)
16	None
17	V_β7.1 (111)
18	None
19	V_β6.7 (123), V_β9 (200)
20	V_β5.1 (157)
21	V_β5.1 (132)
22	V_β5.1 (173), V_β6.7 (229)
23	None
24	V_β5.1 (139), V_β7.1 (213)
25	V_β5.1 (166), V_β9 (191)
26	None

NOTE. Data are V_β receptor (% increase in expression over unstimulated cells). V_α or V_β was considered enhanced if no. of positive cells after exposure to soluble toxoplasma tachyzoite antigen was ≥2-fold higher than the no. of cells bearing the same V_α or V_β in cultures without stimulation.

Discussion

We have shown that patients with acquired ocular toxoplasmosis have higher T cell responses on stimulation with toxoplasma antigens than do seropositive asymptomatic persons. In addition, persons with acquired toxoplasmosis secreted higher amounts of IL-1, TNF-α, and IL-10 than did the asymptomatic group. The synthesis of such cytokines elicited by the parasite in the ocular tissues could account in part for the pathogenesis of the lesions. In addition, our data suggest that resistance to infection may be linked to the ability to secrete high levels of IL-12. These data are in line with previous results in the animal model of toxoplasmosis, showing that susceptible mouse strains express high levels of cytokine mRNAs in the eye [12, 13, 23]. Interestingly, patients with a diagnosis of congenital ocular toxoplasmosis had significantly lower lymphocyte proliferative responses and delayed-type hypersensitivity skin reactions and secreted significantly less IL-2 and IFN-γ in

response to STAg than did patients with a diagnosis of acquired ocular toxoplasmosis. The diminished response to toxoplasma antigens by T cells from patients with congenital disease suggests that *T. gondii*–specific T cells could have been deleted or anergized through exposure to toxoplasma antigens during the prenatal period. Analysis of expression of the V_β families in T cells from these patients showed that the deletions in the T cell repertoire were often shared by members of the same family who did not have congenital toxoplasmosis. Although we cannot rule out deletion as the cause of the decreased T cell responses, our data suggest that anergy may play an important part in the unresponsiveness observed in T cells from patients with congenital disease. Our findings are important because they provide a laboratory tool to differentiate between acquired and congenital disease, and they also suggest that the mechanisms involved in the development of ocular lesions may be different in the two forms of disease, despite the similarity in the pathologic features.

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